

**VALIDATION OF RADIOCHEMICAL PURITY ANALYSIS METHODS USED IN  
TWO TERTIARY PUBLIC HOSPITALS IN SOUTH AFRICA**

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## **DECLARATION**

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## ABSTRACT

Radiopharmaceutical kits are supplied by manufacturers with package inserts containing information about the kit including validated methods of preparation and radiochemical purity (RCP) analysis. Validated analytical methods are also described in pharmacopoeial monographs. However the information provided is not always complete or practical and in a hospital setting it can be difficult to select and perform adequate RCP testing on the prepared radiopharmaceuticals. This situation has led to modifications or substitution for much quicker, simplified, safe or cost-effective analytical procedures. A number of these procedures have been proposed in published literature and have been incorporated in some hospital settings including radiopharmacies in Africa. Since the responsibility of any method that deviates from the official pharmacopoeial or manufacturer's method rests with the end user, this study was aimed to determine whether appropriate validation procedures based on the Q2A and Q2B guidelines of the International Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) are feasible for use in a resource limited environment, which pertains in most hospital radiopharmacy settings in Southern Africa. A further aim was to develop a prototype protocol for validation of analytical procedures in a hospital radiopharmacy setting.

In an attempt to undertake a full analytical method validation, eight validation parameters described in the ICH guidelines were selected for the current study namely specificity, accuracy, precision (repeatability and intermediate precision), linearity, range, limit of detection, limit of quantitation and robustness.

To undertake the validation exercise, fast RCP test methods for Tc-99m sestamibi involving the use of Whatman 31ET and Schleicher and Schuell chromatography paper were used. Locally procured Macherey-Nagel (MN) Alox N aluminium oxide TLC strips were intended as control method as the Baker-Flex aluminium oxide TLC strips described in the manufacturer's instructions could not be sourced. All the tests were performed in triplicate and results were compared. A limited number of tests was also performed on Baker-Flex TLC strips to compare with the results of the substitute MN Alox method. The radiochemical components namely Tc-99m sestamibi, Tc-99m colloid and pertechnetate that were prepared in house and were assumed to be 100 % pure, were each tested on the chromatography strips. Samples containing mixtures of varying concentrations of the radiochemical components were also tested on all the strips.

Radiochemical purity test results of sestamibi samples without any added impurities were  $99.8\% \pm 0.0\%$  for Macherey-Nagel Alox TLC,  $99.5\% \pm 0.1\%$  for Whatman 31ET paper and  $99.3\% \pm 0.2\%$  for Schleicher and Schuell paper chromatography strips. When Tc-99m pertechnetate and Tc-99m colloid were added to Tc-99m sestamibi as impurities after completion of kit reconstitution, the values for sestamibi were in all cases higher than the calculated RCP. These higher results could have been due to binding of the added technetium to the sestamibi. Another possibility would be that another technetium compound was formed after mixing the already prepared radiochemical components. This new impurity then co-migrated with the Tc-99m sestamibi on the chromatography strips. The unknown impurity could not be isolated or quantified. This impurity could not be proved to be Tc-99m pentamibi, as it was not possible to prepare this radiochemical component in-house and hence all the analytical methods lacked specificity. The MN Alox test method showed exceptionally high values for sestamibi due to co-elution of the free pertechnetate with sestamibi in addition to the unknown impurity. As a result, the MN Alox RCP test method could not be used as a reference standard. The poor agreement between the nominal (calculated) and observed results had a negative effect on the accuracy and linearity over the range that was selected of all the three analytical procedures. Apart from meeting the acceptance criteria for repeatability and intermediate precision, all three analytical methods were also noted to be robust. For the radiochromatogram scanner, the limit of detection was 59 counts while the limit of quantitation was 177 counts for the scanning speed and distance used.

In conclusion, all the eight ICH validation parameters are essential when validating a RCP test method. Also, validating an analytical procedure in a hospital setting is possible once some important prerequisites are met, such as availability of staff trained in radiopharmacy or radiochemistry, availability of specified materials for the reference procedure or control experiments, in house preparation of reference standards, and a template validation protocol for thin layer chromatography (TLC) and paper chromatography. Availability of specialized equipment such as a high performance liquid chromatography (HPLC) system for radiopharmaceuticals that have impurities other than free pertechnetate and colloid, is also a requirement, but HPLC is not currently available in public sector Radiopharmacies in South Africa.

## OPSOMMING

Radiofarmaseutiese kitsstelle word deur vervaardigers met voubiljette wat inligting oor die kitsstel bevat, voorsien. Dit sluit bereidingsmetodes en analisemetodes om radiochemiese suiwerheid te bepaal, in. Gevalideerde analitiese metodes word ook in monografieë in farmakopieë beskryf. Die inligting wat voorsien word, is egter nie altyd volledig of prakties nie en in 'n hospitaalopset kan dit moeilik wees om geskikte radiochemiese analise van die bereide radiofarmaseutika te doen. Hierdie situasie het tot aanpassings van metodes of vervanging met vinniger, vereenvoudigde, veilige of koste-effektiewe analitiese prosedures gelei. 'n Aantal van die prosedures is gepubliseer en word in hospitaal radiofarmasiepraktyk, ook in Afrika, toegepas. Die verantwoordelikheid vir enige metode wat van die amptelike farmakopieë of vervaardiger se afwyk, lê by die eindgebruiker daarvan. Hierdie studie het daarom ten doel gehad om te bepaal of toepaslike validasieprosedures gebaseer op die ICH (International Conference on Harmonisation) se Q2A en Q2B riglyne uitvoerbaar is in omgewings met beperkte hulpbronne, soos in hospitaal radiofarmasiepraktyke in suidelike Afrika. 'n Verdere doelstelling was om 'n prototipe protokol vir die validering van analitiese prosedures in hospitaalradiofarmasie te ontwikkel.

In 'n poging om 'n volle analitiese validering volgens ICH riglyne te doen, is agt validasie parameters, naamlik spesifisiteit, akkuraatheid, presisie (herhaalbaarheid en intermediêre presisie), lineariteit, reikwydte, perk van opsporing, perk van kwantifisering en robuustheid, vir hierdie studie gekies.

Vinnige radiochemiese analisemetodes vir die evaluering van Tc-99m sestamibi is gebruik om die valideringsproses te toets. Whatman 31ET en Schleicher en Schuell chromatografie papier is gebruik. Plaaslik verkrygbare Macherey-Nagel (MN) Alox N aluminiumoksied dunlaagstrokies was aanvanklik die keuse van kontrolemetode, aangesien die Baker-Flex aluminiumoksied strokies wat die kitsstelvervaardiger se aanwysings voorskryf, nie verkry kon word nie. Alle toetse is in drievoud gedoen en resultate is vergelyk. 'n Beperkte aantal toetse is aan die einde van die studietydperk met Baker-Flex TLC strokies uitgevoer om dit met die plaasvervangende MN Alox aluminiumoksied te vergelyk. Die radiochemiese komponente, naamlik Tc-99m sestamibi, Tc-99m kolloïed en vry pertegnetaat is intern berei met veronderstelde suiwerheid van 100 %. Al drie komponente is individueel op die chromatografiestrokies getoets, asook mengsels met wisselende konsentrasies van die drie vorms van Tc-99m.

Die gemete radiochemiese suiwerheid van sestamibi monsters sonder enige bygevoegde onsuierhede was  $99.8 \% \pm 0.0 \%$  vir Macherey-Nagel Alox strokies,  $99.5 \% \pm 0.1 \%$  vir Whatman 31ET papier en  $99.3 \% \pm 0.2 \%$  vir Schleicher en Schuell papier chromatografie strokies. Toe Tc-99m pertegnetaat en Tc-99m kolloïed as onsuierhede na kitsstelbereiding by Tc-99m sestamibi gevoeg is, was die waardes vir sestamibi in alle gevalle hoër as die berekende radiochemiese suiwerheid. Hierdie hoër waardes mag moontlik te wyte wees aan binding van die bygevoegde tegnesium aan die sestamibi. Nog 'n moontlikheid mag wees dat 'n ander tegnesium verbinding na vermenging van die verskillende radiochemiese komponente gevorm is. Die nuwe onsuierheid het dan saam met sestamibi op die chromatografie strokies migreer. Die onbekende onsuierheid kon nie geïsoleer of kwantifiseer word nie. Dit kon ook nie aangetoon word of dit Tc-99m pentamibi was nie, aangesien dit nie moontlik was om hierdie radiochemiese spesie plaaslik te berei nie. Al die analitiese metodes was dus nie spesifiek genoeg nie. Die MN Alox metode het buitengewoon hoë waardes vir sestamibi getoon as gevolg van ko-migrasie van vry pertegnetaat met sestamibi bykomend tot bogenoemde onbekende onsuierheid. Die MN Alox radiochemiese analisemethode was dus nie as verwysingsmetode geskik nie. Die swak korrelasie tussen die teoretiese (berekende) en gemete resultate het die akkuraatheid en lineariteit oor die gekose reikwydte vir al drie analisemetodes negatief beïnvloed. Al drie metodes het aan die kriteria vir herhaalbaarheid en intermediêre presisie voldoen en was robuus. Daar is getoon dat die radiochromatogram skandeerder se perk van opsporing (limit of detection) 59 tellings en perk van kwantifisering (limit of quantitation) 177 tellings vir die gebruikte skandeerspoed en lengte was.

Die gevolgtrekking van hierdie studie is dat al agt ICH valideringsparameters noodsaaklik is wanneer 'n analisemethode vir radiochemiese suiwerheid evalueer word. Validering van 'n analitiese metode in 'n hospitaalopset is moontlik, mits aan 'n paar belangrike vereistes voldoen kan word. Voorbeelde hiervan is beskikbaarheid van personeel wat in radiofarmasie of radiochemie opgelei is, beskikbaarheid van die gespesifiseerde materiaal vir die verwysingsmetode of kontrole eksperimente, die moontlikheid om verwysingstandaarde te berei, asook beskikbaarheid van 'n templaar valideringsprotokol vir dunlaag- en papierchromatografie. Beskikbaarheid van gespesialiseerde apparaat soos 'n HPLC sisteem vir radiofarmaseutiese produkte met ander onsuierhede as vry pertegnetaat en kolloïed, is ook 'n vereiste, maar HPLC is oor die algemeen nie in hospitaalradiofarmasiepraktyke in Suid-Afrika beskikbaar nie.

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## **DEDICATION**

THIS WORK IS DEDICATED TO GOD AND MY FAMILY



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## LIST OF ABBREVIATIONS

FDA:	Food and Drug Administration
GLP:	Good Laboratory Practice
GMP:	Good Manufacturing Practice
HPLC:	High Performance Liquid Chromatography
ICH:	International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use
ITLC-SG:	Instant thin layer chromatography-silica gel
LE:	Labeling efficiency
LOD:	Limit of detection
LOQ:	Limit of quantitation
MBq:	Megabecquerel
MIBI:	Methoxy-isobutyl-isonitrile
MPC:	Mini paper chromatography
NRC:	Nuclear Regulatory Commission
RCP:	Radiochemical purity
R <sub>f</sub> :	Relative front
S <sub>f</sub>	Solvent front
SPC:	Summary of Product Characteristics
SPE:	Solid-phase extraction
Tc-99m:	Technetium-99m
TLC:	Thin Layer Chromatography

## CHAPTER ONE

### GENERAL INTRODUCTION

Radiochemical purity (RCP) analysis is an important analytical procedure in Radiopharmacy. The analysis determines how much of the radioactivity in a radiopharmaceutical is in the desired chemical form. Manufacturers of radiopharmaceutical kits provide information on a validated analytical method to be performed on their products. However, some prescribed methods tend to have directions that are not clear, some procedures take a long time and some may involve the use of expensive equipment or solvents that may not be safe to handle. Alternative analytical methods are used in many radiopharmacies in Africa where resources are often limited. If more cost effective analytical methods or simple, quicker methods are adopted, they should provide reliable results.

Studies have been published revealing shortfalls in some of the alternative methods which would result in incorrect results which may in turn lead to an erroneous diagnosis. While some researchers recommend that methods for RCP analysis should be viewed as guidelines and not as requirements, giving room for modifications to be made on the method, responsibility of the results of deviating from or modifying the recommended method rests with the end-user. It is therefore important that the analytical procedure be validated against the manufacturers' or pharmacopoeial method.

The current study aimed to determine whether validation of RCP analysis of radiopharmaceuticals was feasible in hospital settings in Southern Africa, despite being in a resource limited environment. Tc-99m sestamibi was used as a model for the study where alternative RCP methods involving the use of Whatman 31ET as well as Schleicher and Schuell chromatography paper, currently being used at Tygerberg and Groote Schuur Hospitals respectively were validated against Macherey-Nagel (MN) Alox N aluminium oxide TLC strips. The MN Alox strips were used as they could be locally procured since the manufacturer's recommended Baker-Flex aluminium oxide TLC strips were not locally available. Validation parameters described in ICH Q2A and Q2B guidelines which are used in the pharmaceutical industry were used for the validation process.

## CHAPTER TWO

### LITERATURE REVIEW

#### Validation in Pharmaceutical Manufacture

Analysis of pharmaceutical products is an essential part of quality assurance in pharmacy <sup>[1]</sup>. Analytical procedures are performed during each stage of drug manufacture and assess the quality of the product. The analytical procedures help to ensure that the precursors, intermediates and the finished product meet the required specifications in order to assure that the final product is safe and effective <sup>[1-5]</sup>. The most common types of analytical procedures that are used in pharmaceutical manufacture are categorized into four assay groups namely identification tests, quantitative tests for impurities, limit tests for the control of impurities and quantitative tests of the active moiety in the sample of the drug substance or drug product, or other selected components in the drug product <sup>[1-4, 6-8]</sup>. Identification tests help to identify the analyte in the sample by comparing it with a known standard. The quantitative test or limit test for impurities in a sample helps to determine the purity characteristics of the sample. Assay tests quantitatively measure the major component(s) in the drug substance.

Results of analysis can only be reliable if the analytical method employed is proven to be able to give truthful results. It is therefore important to ensure that the analytical procedure used is validated. Validation of analytical procedures is defined as the process by which it is established by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use <sup>[1]</sup>. Validating an analytical procedure helps to identify any errors or shortfalls and enables appropriate measures to be taken to improve the procedure <sup>[8, 9]</sup>. This increases the value of the procedure and makes it more reliable as the results can be used to judge the quality, reliability and consistency of the analytical results <sup>[3, 5, 7-9]</sup>. Ideally, analytical methods should be validated when the method is introduced in a laboratory or when conditions or parameters of the original method change <sup>[1, 2, 4, 8-10]</sup>. Instructions on how to validate an analytical procedure are provided in a validation protocol or validation master plan and results are presented in a validation report <sup>[1, 2, 7, 8, 11]</sup>. The importance of validating an analytical procedure is emphasized by a number of guidelines set up by regulating bodies, published literature and pharmacopoeias. Validation of analytical procedures forms part of regulatory requirements, good science, good manufacturing practice (GMP) and good laboratory practice (GLP) <sup>[2-4, 6-8, 11-14]</sup>.

## Parameters for validation of analytical procedures

In the pharmaceutical industry, analytical techniques for validation are well described. The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) developed guidelines for the pharmaceutical industry. These include guidelines for the validation of analytical procedures, known as the ICH Q2A and Q2B guidelines. The parameters used to validate an analytical procedure include specificity, accuracy, precision, linearity, range, detection limit, quantitation limit and robustness [1, 2, 4, 6-11, 13-15].

**Specificity** is the ability to assess unequivocally the analyte in the presence of other components which may be expected to be present. This investigation is carried out to identify the compound of interest, to accurately state the impurities that might be present, and assay the main active ingredient (identify exact content or potency in a sample). For chromatograms, it is important to know the peak or retention time of the target compound as it is not always possible to know how many compounds might be present in the sample. The procedure should also be able to separate analytes that might be present in different forms such as free, complexed, organic, or organometallic form or in different oxidation states. [1, 2, 4, 6-11, 13-15]

**Accuracy** expresses the closeness of agreement between the true value and the value found in analysis. This investigation helps to show the extent to which the tests results and the true value agree. The accuracy of an analytical procedure can be determined either by comparing results from a test method with those of an established reference method or by comparing test results with standards whose concentrations are known. [1, 2, 4, 6-11, 13-15]

The **precision** of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of samples under prescribed conditions. There are three types of precision tests namely intermediate, repeatability and reproducibility precision. Intermediate precision typically involves tests that are performed in the same laboratory but on different days, by different analysts and using different equipment. For repeatability, tests are carried out in the same laboratory within short intervals of time and by the same operator using the same equipment. Reproducibility involves tests that are carried out by different operators, using different equipment and in different laboratories. [1, 2, 4, 6-11, 13-15]

**Linearity** is the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. This can be achieved by diluting a stock solution or by separately weighing samples of a test product. Some guidelines recommend that five concentrations be used for the establishment of linearity while others recommend between 80 and 120 percent of the expected concentration range. Linearity can be evaluated graphically by visually inspecting a plot of signal (peak height or peak area) as a function of analyte concentration. [1, 2, 4, 6-11, 13-15]

The **range** is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. [1, 2, 4, 6-11, 13-15]

The **detection limit** is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. It is the point at which the value of the analyte is larger than the uncertainty associated with it. The limit of detection can be determined from visual evaluation, standard deviation of the response based on the standard deviation of the blank and the standard deviation of the response based on the slope of the calibration curve. [1, 2, 4, 6-11, 13-15]

The **quantitation limit** is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. This parameter is useful in qualitative assays for samples with low level compounds such as determination of impurities or degradation products. For chromatographic methods, in order to establish the lowest amount of analyte that can be quantitated reliably, signals from samples with known concentrations of analyte are compared with those of blank samples. Spiked matrices that closely represent the unknown samples can be used in addition to using pure standards. [1, 2, 4, 6-11, 13-15]

**Robustness** is a measure of the method's capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. This parameter is normally evaluated during development of the procedure. For each procedure, it is important to identify stages that can have severe effects on the performance of the procedure if carried out in less than perfect conditions. The information acquired from robustness tests can guide on the specific conditions under which the analytical procedure needs to be carried out and if need be a precautionary statement can be included in the procedure. [1, 2, 4, 6-11, 13-15]



## Analysis of radiopharmaceuticals in Nuclear Medicine

Technetium-99m (Tc-99m) radiopharmaceuticals are used for many diagnostic procedures in Nuclear Medicine <sup>[16-21]</sup>. The components are supplied by specialized manufacturing companies in the form of radionuclide generators and nonradioactive reagent kits. The radiopharmaceutical is then prepared at the Nuclear Medicine department by adding Tc-99m to a tracer molecule in order to form a radiopharmaceutical. After preparation, the radiopharmaceutical administered to the patient is expected to localize in a particular organ or system of interest in the body. Radiochemical impurities such as free pertechnetate and hydrolysed reduced technetium can also be present if not all of the radionuclide binds to the pharmaceutical or reagent. Other forms of impurities for example Tc-99m pentamibidimethylvinylisonitrile <sup>[22]</sup> in Tc-99m sestamibi and Tc-99m tartrate in Tc-99m mercaptoacetylglycylglycylglycine (Tc-99m MAG3) <sup>[23]</sup> may also be present.

Free pertechnetate can be present as a result of the presence of oxygen in the vial, which can oxidize the stannous ion to stannic ion thereby decreasing the stannous ion available to reduce the oxidation state of Tc-99m. Hydrolyzed reduced technetium is formed as a result of reaction of the reduced Tc-99m with water. The presence of radiochemical impurities in a radiopharmaceutical injected into the patient results in the activity localizing in areas other than the organ or system of interest. This can affect the interpretation of the scan, making it difficult to derive a proper diagnosis. <sup>[16-21]</sup>

For radiopharmaceuticals prepared in Nuclear Medicine, the efficacy of the final product is highly dependent on the strict adherence to the method of preparation. So in addition to a number of quality control tests that are carried out by the manufacturers on cold kits, some quality control tests are also performed on the radiopharmaceuticals in the Nuclear Medicine departments just before they are administered to patients to ensure that the radiopharmaceutical is correctly radiolabeled <sup>[16-21]</sup>. This means that the Nuclear Medicine department staff members are also responsible for the quality control of the product.

For Tc-99m labeled radiopharmaceuticals, analytical procedures are aimed to identify and quantify not only the radiopharmaceutical in the desired chemical form, but are also aimed at identifying impurities that might be present. Determination of the radiochemical purity (RCP) is an example of such a quality control method used in radiopharmacy. RCP is defined as the fraction of the total radioactivity in the desired chemical form in the radiopharmaceutical. <sup>[16-21]</sup>

The common types of RCP measurement methods that are used include paper, thin layer, column and high performance liquid chromatography (HPLC)<sup>[15-21]</sup>. For paper and thin layer chromatography, a small drop of the sample is placed at the origin on the paper or thin layer strip (stationary phase), which is then placed vertically in a developing chamber containing an appropriate solvent (mobile phase). Each radiochemical component present in the sample interacts differently with the stationary and mobile phases<sup>[17, 19]</sup>. Separation takes place as each component travels up to a characteristic distance while the solvent migrates along the stationary phase up to the desired distance. The distance covered by the solvent is referred to as the solvent front ( $S_f$ ) while the relative front ( $R_f$ ) is the ratio between the distance travelled by a particular radiochemical component to the distance travelled by the mobile phase<sup>[17,19]</sup>. The  $R_f$  values assist the analyst to divide the chromatography strip into sections or regions of interest (ROIs) where the peak area for each radiochemical component present in the sample is expected to localise. Once the solvent has run up to the desired distance, the strip is dried and then measured in an appropriate counter such as a well scintillation counter, dose calibrator or a radiochromatogram scanner. Pertechnetate is able to migrate with the solvent front in polar solvents (such as acetone, saline) while in non-polar lipophilic solvents (such as ethyl acetate and chloroform) pertechnetate is retained at the origin<sup>[20]</sup>. Colloids are retained at the origin in most systems since they are insoluble<sup>[20]</sup>. Personnel performing the analysis should have adequate knowledge of the chemistry of the radiolabeling process in order to prevent errors in RCP analysis.

For high performance liquid chromatography (HPLC), the sample is injected via an injection valve under pressure into a column which is packed with appropriate packing materials. Although the principles for HPLC are similar to those of paper and thin layer chromatography, HPLC is able to separate the different components at high resolution giving it an advantage over paper and thin layer chromatography (TLC) as a better distinction between the different substances is possible. With appropriate detection techniques, all components can be observed with TLC, however it is not as accurate or as sensitive as HPLC. HPLC also allows chemical quantitation and quantification of components.<sup>[15-21]</sup>

Column chromatography is also another commonly used method. It not only enables separation of a mixture into different components, but also allows collection of the separated components. In radiopharmacy, solid-phase extraction (SPE) is commonly used and has been shown to be quite sensitive, simple and reasonably fast<sup>[21]</sup>. SPE uses a solid phase and a liquid phase to separate a compound of interest present in a sample. The solution is first

loaded onto the SPE solid phase and is followed by first washing off the impurities and then washing off the compound of interest by appropriate solvents. <sup>[17, 21, 24]</sup>

RCP analytical methods for various radiopharmaceuticals are described in pharmacopoeia and package inserts or Summaries of product characteristics (S.P.C.) <sup>[12, 22-27]</sup>. For a number of radiopharmaceuticals however, instructions in package inserts tend to be unclear or incomplete <sup>[16, 28]</sup>. A study carried out by Hung et al <sup>[28]</sup> identified a number of deficiencies in the instructions given in package inserts of commonly-used radiopharmaceuticals. Instructions tended to be incomplete or absent, some were restrictive, inconsistent, impractical and vague. Studies by Hung et al. <sup>[29, 30]</sup>, Luebke et al. <sup>[31]</sup> and Proulx et al. <sup>[32]</sup> observed that some recommended analytical methods were time consuming and required the use of expensive equipment. This factor has led to research studies to try and modify or devise alternative analytical methods that are simpler, quicker and cheaper <sup>[33]</sup>. The modified methods were reported to be more practical to implement in daily routine operations in many radiopharmacies. Hung et al <sup>[28]</sup> recommended that instructions in package inserts be viewed as standard guidance and not as required procedures, in order to provide room for the laboratories to modify if need arises. A study by Ballinger et al <sup>[34]</sup> found that some radiopharmacies in the United Kingdom viewed RCP analysis as not necessary to perform each time a radiopharmaceutical is prepared. In every situation described above, the reliability of any analytical method must be proven to give truthful results by ensuring that it is validated.

### **Technetium-99m sestamibi**

Tc-99m sestamibi (chemical name (OC-6-11)-hexakis[1-isocyano-κC-2-methoxy-2-methylpropane][<sup>99m</sup>Tc]technetium(1) chloride)<sup>[12]</sup> is an imaging agent that is used in the diagnosis of ischemic heart disease, diagnosis and localization of myocardial infarction, assessment of ventricular function and localization of hyper-functioning parathyroid tissue <sup>[16, 19, 20]</sup>. Tc-99m sestamibi preparation involves adding pertechnetate solution to the vial containing the chelating agent and the mixture is then heated in a boiling water bath for about 10 minutes. After allowing the preparation to cool for 15 minutes, RCP analysis should be performed on the product before it is administered to the patient <sup>[16, 19, 20, 22, 25 - 27]</sup>.

The analytical procedure is aimed to separate Tc-99m sestamibi from the impurities that might be present in the sample such as free pertechnetate, Tc-99m colloid and Tc-99m pentamibidimethylvinylisonitrile<sup>[22]</sup> ((OC-6-22)-pentakis [1-(isocyano-κC)-2-methoxy-2-

methylpropane][1-(isocyo- $\kappa C$ )-2methylprop-1-ene[99mTc]technetium(1+))<sup>[12]</sup>. By separating Tc-99m sestamibi from the impurities present in the preparation, the labeling efficiency can be determined. A labeling efficiency of 90 % and above should be achieved if sestamibi is prepared in accordance with the manufacturer's instructions<sup>[12, 19, 22, 25, 26]</sup>. After preparation, Tc-99msestamibi remains stable for six hours<sup>[19, 21]</sup>. Studies by Cooper et al.<sup>[35]</sup> and Thomson et al.<sup>[36]</sup> have shown that modifications to the method of radiopharmaceutical preparation affected the labeling process thereby increasing the amount of impurities in the product. Several other factors, either alone or in combination also affected the radiolabeling process such as generator growth or amount of Tc-99m formed between the previous and current elution, the amount of activity added to the reagent kit, generator manufacturer, eluate age and heating temperature and duration<sup>[37-40]</sup>.

Baker-Flex aluminium oxide-coated plastic thin layer chromatography medium as stationary phase and ethanol ( $\geq 95\%$ ) as the mobile phase are recommended by manufacturer<sup>[25, 26]</sup> for radiochemical purity analysis of Tc-99m sestamibi. Another RCP method is described in the European Pharmacopeia<sup>[12]</sup>. This method involves a combination of three analyses, namely thin layer chromatography, paper chromatography and high performance liquid chromatography (HPLC), all with mobile phase mixtures containing at least three solvents. The combination of these 3 analyses is used to identify three impurities found in Tc-99m sestamibi preparations. A similar method is also described in the United States Pharmacopoeia (USP)<sup>[22]</sup> which involves the use of thin layer chromatography and HPLC. Some limitations to the manufacturer's prescribed method were noted when modifications were made to the method of radiopharmaceutical preparation. A study by Thomson et al.<sup>[36]</sup> found that the manufacturer's method overestimated the RCP, due to failure to separate Tc-99m sestamibi from two highly lipophilic impurities whose retention time on HPLC resembled that of pertechnetate. But a study by Cooper et al.<sup>[35]</sup> revealed impurities that did not migrate with the acetone when similar samples were used on instant thin layer chromatography-silica gel (ITLC-SG) strips suggested by Proulx et al<sup>[32]</sup>.

Due to the short physical half-life of Tc-99m, it is important to have an analytical procedure that can be performed within the shortest possible time but at the same time, be able to provide the maximum amount of information<sup>[19, 20]</sup>. For Tc-99m sestamibi, the RCP method recommended by the manufacturer tends to be time consuming and therefore a number of studies have been undertaken where alternative methods that are faster and fairly easy to perform have been proposed<sup>[33]</sup>. Alternative methods involving the use of Whatman 3MM

paper<sup>[41]</sup>, HPLC<sup>[42]</sup> and a silica gel for instant thin layer chromatography ITLC-SG<sup>[32]</sup> were found to be effective, reliable and fast in determining the RCP of Tc-99m sestamibi. Whatman 31ET paper<sup>[31, 33]</sup> and Gelman solvent saturation pads<sup>[29]</sup> were also found to be fairly easy to use.

Despite being more practical, the alternative RCP methods also have their limitations. A number of problems were observed with paper chromatography using Whatman 31ET. One study by Hung<sup>[43]</sup> observed streaking while Luebke et al<sup>[31]</sup> noted that a falsely elevated labeling efficiency of up to 33% for the regular and 27% for the mini Whatman 31ET strip was observed for labeling efficiency results that were less than 90%. Another study by Masarat et al.<sup>[44]</sup> showed that only small developing chambers were able to give high labeling efficiencies. They also took less time to develop and showed better separation between the impurities and Tc-99m sestamibi.

Radiopharmaceuticals are regarded as medicines and hence their production and use are subject to regulations<sup>[45, 46, 47]</sup>. In the pharmaceutical manufacturing industry, regulatory bodies establish guidelines on various aspects of drug manufacture including validation of analytical procedures<sup>[1, 5, 46]</sup>. Many of these are derived from the International Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines<sup>[7]</sup>. In analytical method validation, the ICH has provided the Q2A and Q2B guidelines<sup>[6, 14]</sup> to industries that intend to validate their analytical methods. Although his movement is meant to provide guidance on the scientific and technical aspects needed to register a product, a number of gaps or limitations have been noted<sup>[45]</sup>. The ICH does not provide specific information for validating RCP analytical methods for radiopharmaceuticals prepared in hospital settings. In the United States of America, radiopharmaceutical production and use is regulated by the Food and Drug Administration (FDA) and by the Nuclear Regulatory Commission (NRC)<sup>[16, 19, 47]</sup>. The FDA has provided extensive regulations and guidelines in the pharmaceutical manufacturing industry but many of the regulation requirements including specific recommendations on how to validate analytical procedures have not been applicable to radiopharmaceuticals. Despite the establishment of the Radioactive Drug Research Committee (RDRC) (21CFR361.1) to specifically regulate radiopharmaceutical manufacture, this has not been able to fully address all the gaps present. In Europe, the EANM<sup>[46]</sup> guidelines and directives have provided limited information on RCP analytical method validation.

With no specific guidelines available, slight variations in the validation process have been described in published literature that proposed alternative analytical methods. Hung et al <sup>[29, 30]</sup> and Luebke et al <sup>[31]</sup> mainly compared the accuracy linearity and specificity. While Leonardi et al <sup>[51]</sup> and Seetharaman et al <sup>[52]</sup> validated in line with ICH guidelines, they did not find it necessary to include limit of detection (LOD) and limit of quantitation (LOQ) parameters due to properties of the radiation detecting equipment and also since only RCP values of 90 % and above are used in clinical studies. However, similarities in the experimental designs are observed<sup>[29-32]</sup>. Hung et al <sup>[29,30]</sup>, Luebke et al <sup>[31]</sup> and Proulx et al <sup>[32]</sup> tested proposed alternative methods simultaneously with a pharmacopoeial or the manufacturer's already validated method. In these studies, the radiopharmaceutical was prepared in accordance to the prescribed method. The analyst then created samples with different, lower RCP results over a particular range. Each analytical method was performed according to its prescribed instructions and results were then compared<sup>[29-32]</sup>. While many studies provided information on experimental methods and results, some however, have not published this information. Zimmer et al <sup>[33]</sup> did not provide any information on the experiments that were performed or results for the alternative RCP test methods that were proposed in their summary of miniature chromatography.

In summary, analytical procedures are important in the pharmaceutical industry to ensure that drug products that are produced are safe and effective. Although official and validated analytical methods are available for radiopharmaceuticals, a number of radiopharmacies and Nuclear Medicine practices prefer to use alternative methods which tend to be easier to use. General pharmaceutical guidelines recommend that all new analytical methods introduced to a laboratory must first be validated. However, since currently there are limited guidelines for radiopharmaceutical validation, the question that arises is, how RCP analysis can be validated and if it is possible to perform adequate validation in a hospital radiopharmacy setting without good analytical facilities and equipment.

## AIM OF THE INVESTIGATION

The aim of this study was evaluate the feasibility of validating radiochemical purity analysis methods in hospital radiopharmacies, using Tc-99m sestamibi and two alternative radiochemical purity (RCP) analytical methods used in two tertiary hospitals in Southern Africa as a model.

Two specific objectives of this study were

1. To validate the analytical methods using eight parameters namely accuracy, precision (repeatability and intermediate), linearity, range, specificity, limit of detection, limit of quantitation and robustness, based on ICH Q2A and Q2B guidelines and,
2. To recommend a preliminary protocol template for validation of RCP analytical procedures in tertiary hospital settings.

## CHAPTER THREE

### MATERIALS AND METHODS

Approval to undertake this study was obtained from the Stellenbosch University Health Research Ethics Committee (No: S12/ 11/ 315). The study entailed purely laboratory analytical work and did not involve human subjects. For the study, RCP analytical methods used at Tygerberg and Groote Schuur Hospitals for Tc-99m sestamibi were both evaluated at Tygerberg Hospital. Permission to undertake this study was obtained from Tygerberg Hospital management.

#### Study population:

Two alternative analytical methods to determine the RCP of Tc-99m sestamibi were evaluated. The method used at Tygerberg Hospital employed Whatman 31ET Chr Cellulose Chromatography paper (GE Healthcare Life Sciences, Pittsburgh, USA, product code 3031-915) with ethyl acetate as mobile phase. This method is referred to as the Whatman 31ET method in the remainder of this document. The method proposed by Groote Schuur Hospital used Schleicher and Schuell No. 2040a (Hahnemühle FineArt, GmbH, Dassel, Germany) chromatography paper strips with ethyl acetate. As reference method, Polygram® Alox N strips (Macherey-Nagel GmbH & Co, Düren, Germany, ref 802 012) with ethanol was used, as the Baker-Flex aluminium oxide strips (J.T. Baker® by Avantor performance Materials, Center Valley, USA, catalogue JT4467-4) recommended by the kit manufacturers were not available during the initial study period.

For each sample that was prepared, analyses were performed using all three RCP analytical methods, with a total of 159 samples that were analysed between February 2013 and August 2014.

#### Radiopharmaceutical preparation

Tc-99m sestamibi was prepared by adding 12000- 13000 MBq (activity measured using a CAPINTEC CII®CRC-15R dose calibrator) of pertechnetate in a volume of 3 ml obtained from the NovaTec-P Technetium-99m generator (NTP, South Africa) to a sterile pyrogen free MIBI kit (Stamici®, CIS bio international). The radiopharmaceutical was then heated in a water bath for 10 minutes and allowed to cool for 15 minutes. In most cases, the samples for



analysis were obtained from the vials prepared for patient doses which were all assumed to be 100 % pure.

For the colloid impurity, tin colloid kits (NTP, South Africa) were used. The colloid was prepared by adding 3400-3700 MBq of pertechnetate in a volume of 1ml to the kit and the kit was allowed to stand for 15 minutes.

Pertechnetate was obtained from the NovaTec-P generator and diluted to approximately 4000 MBq/ml.

#### Chromatography strip preparation and procedure for radiochemical purity analysis

MN Alox method: Aluminium oxide (0.20 mm aluminium oxide on polyester sheets, Macherey-Nagel POLYGRAM® ALOX N) coated strips were cut to 2.5 cm x 7.5 cm and marked with a pencil to identify the origin 1.5 cm from the bottom and the solvent front 5cm from the origin. The strips were then dried in an incubator (ENKAB®-1, England) at a temperature of 70-74°C for 1-2 hours. When performing radiochemical purity analysis, a drop of ethanol was spotted at the origin, 1.5 cm from the bottom. Two drops (2 µl each using a Hamilton Syringe (Hamilton-Bonaduz, Schweiz)) of Tc-99m sestamibi were immediately added next to each other on top of the ethanol spot. After allowing the sample spot to dry, the Alox strip was then placed in the developing chamber with a cover containing about 3-4 mm of absolute 99% ethanol and allowed to run until the solvent reached the solvent front mark. The Alox strip was removed and allowed to dry and was later scanned on a chromatogram scanner.

Whatman 31ETmethod: Whatman 31ETchromatography strips were cut 0.9 cm x 8.5 cm and marked with a pencil to identify the origin at 1 cm from the bottom, and the solvent front line 6.5 cm from the origin. When performing radiochemical purity analysis, a drop (2 µl) of Tc-99m sestamibi was spotted on the origin line and the strip was immediately placed in the chamber with a lid containing 4 - 5 mm of ethyl acetate and allowed to develop. When the solvent reached the solvent front line, the strip was removed and allowed to dry before being scanned on the chromatogram scanner.

Schleicher and Schuell method: Schleicher and Schuell (2040a) paper strips were cut 2 cm x 7.5 cm and marked with a pencil to identify the origin line at 2 cm from the bottom and front line at 7 cm. After spotting 2 µl of Tc-99m sestamibi, the strip was immediately placed in a developing chamber with a lid and containing 1 ml (4 - 5 mm depth) of ethyl acetate. As soon

as the solvent reached the solvent front, the strip was removed and allowed to dry before being scanned on the chromatogram scanner.

### Chromatogram quantification

After the strips were dried, they were scanned on a radio-chromatogram scanner (Veenstra® VCS-101) using a low energy collimator with a 2 mm slit and an energy window of between 126.0 and 154.0 keV. The scan length was 120 mm with the strip placed 25 mm from the leading edge of the scanner plate which moves past the detector at a speed of 100 mm per minute.

In all three strips, the impurities are noted to appear in the area near the origin while the sestamibi appears in the area close to the solvent front line. The strips are hence divided into regions 1 (the area covered by the impurity peaks) and region 2 (the area covered by the entire peak of the pure Tc-99m sestamibi). These regions were stored in the scanner database and applied to all chromatograms of the relevant method.

**Table 1: Regions selected on chromatograms**

Method	Strip length (mm)	Strip position on scanner plate (mm)	Region 1 (mm)	Region 2 (mm)
MN Alox	75	25 -100	24 - 50	51– 105
Whatman 31ET	85	25 - 110	25 - 55	56 - 115
Schleicher and Schuell	75	25 - 100	25 - 50	51 - 105

### Samples containing sestamibi and impurities:

According to the manufacturers, Tc-99m sestamibi should only be used if the RCP is at least 90 %. Values lower than 90% are seldom seen in routine use of the product. We therefore decided to use a range of RCP values between 80 % and 100 % for the validation experiments. Samples containing known quantities of sestamibi and impurities were prepared. The sestamibi concentrations aimed for in the five mixtures were 80 %, 85 %, 90 %, 95 % and 100 % sestamibi. Freshly prepared and cooled Tc-99m sestamibi was used as 100% sestamibi sample. For each concentration from the selected range, calibrated quantities of sestamibi, colloid and pertechnetate (as shown in the table below) were each taken from the respective kit

vials immediately after radiolabeling and were all added to an empty vial. In practice, it was difficult to prepare exactly identical concentration of the three solutions, but the exact concentration of the separate sestamibi, pertechnetate and colloid solutions were known. The actual concentration of each mixture could thus be calculated.

Samples of varying concentration of Tc-99m sestamibi containing varying concentrations of pertechnetate and Tc-99m colloid were prepared as shown below.

**Table 2: Preparation of sestamibi of different concentrations**

<b>Concentration of MIBI</b>	<b>ml MIBI to be pipetted from original vial (A)</b>	<b>ml of <math>\text{TcO}_4^-</math> to be pipetted from <math>\text{TcO}_4^-</math> vial (T)</b>	<b>ml Colloid to be pipetted from Colloid vial (C)</b>	<b>Total volume</b>
<b>80 %</b>	1.60 ml (6400MBq)	0.20 ml (800 MBq)	0.2 ml (800 MBq)	2 ml
<b>85 %</b>	1.70 ml (6800 MBq)	0.15ml (600MBq)	0.15 ml (600 MBq)	2 ml
<b>90 %</b>	1.80 ml (7200 MBq)	0.1 ml (400 MBq)	0.1 ml (400MBq)	2 ml
<b>95 %</b>	1.90 ml (7600 MBq)	0.05ml (200 MBq)	0.05 ml (200 MBq)	2 ml
<b>100 %</b>	Original solution without diluting	-	-	2 ml

## VALIDATION EXPERIMENTS

### Specificity

To determine specificity, first, Tc-99m sestamibi and each impurity (pertechnetate and Tc-99m colloid respectively) were individually tested on all three analytical methods to identify the location of peaks on the chromatographic strips. Then samples containing known amounts of Tc-99m sestamibi and both impurities were used to determine whether each analytical procedure was able to separate and quantify the individual components present.

Three analyses were performed for each radiochemical component (radiopharmaceutical compound and expected impurities) individually and for the mixtures of sestamibi with impurities using each of the three methods.

#### *Acceptance criteria*

A method was regarded acceptable if it was able to separate and show components present in the sample. This was judged by comparing the  $R_f$  values of the radiopharmaceutical compound and each impurity to verify that there was no overlap of peaks i.e. no co-elution of impurities with the radiopharmaceutical compound.

#### **Accuracy**

To determine accuracy, the observed results obtained from the alternative analytical methods were each compared to the nominal or calculated values of Tc-99m sestamibi. The samples that were used were samples that contained 80.8 %, 90.4 % and 100 % Tc-99m sestamibi.

At each concentration (80.8 %, 90.4 % and 100 % Tc-99m sestamibi), a total of 9 tests (3 replicates at each of the 3 concentrations) were performed for each analytical method over the selected range of  $90\% \pm 10\%$  or pharmacopoeial labeling efficiency limit  $\pm 10\%$ . After obtaining the mean result for each concentration, results obtained for the alternative analytical methods were compared to those the nominal concentration or mean value of the recommended analytical procedure.

#### *Acceptance criteria*

A method was accepted to be accurate when the mean of the measured values for alternative analytical method differed by not more than 2 % from the mean value of the nominal concentration or mean value of the recommended analytical procedure.

#### **Precision**

For precision, each method was assessed individually to judge the closeness of agreement between the measurements. Samples containing 100 % Tc-99m sestamibi were used for this assessment.

For **repeatability**, 6 analyses were performed using only one sample containing 100 % of the radiopharmaceutical compound. The average and the percentage relative standard deviation (% RSD) were calculated using the obtained results using the formula for % RSD:

$$(\text{SD/average}) \times 100$$

#### *Acceptance criteria*

The percentage relative standard deviation (% RSD) had to be 15 % or less.

For **intermediate precision**, two different operators each prepared one sample containing 100 % concentration of the radiopharmaceutical compound. Each operator performed 6 replications on the prepared sample. The average and the percentage relative standard deviation (% RSD) were determined for each set of results. Also, to compare the two groups Student's T-test for independent samples was used.

#### *Acceptance criteria*

The percentage relative standard deviation (% RSD) had to be 15 % or less and for Student's T-tests for independent samples,  $p < 0.05$ .

### **Linearity and range**

Five concentrations (80.8 %, 85.6 %, 90.4 %, 95.2 % and 100 %) sestamibi were selected for the study and linear regression analysis was used to determine the proportionality between the measured and calculated results. This was determined for each analytical method individually and a range  $90\% \pm 10\%$  or pharmacopoeial labeling efficiency limit  $\pm 10\%$  was selected.

#### *Acceptance criteria*

The correlation coefficient ( $r$ ) had to be  $\geq 0.98$ , the Y-intercept had to be  $\leq 5\%$  of the target concentration, and the slope was expected to be 1.

### **Limit of detection and limit of quantitation**

Twenty blank scans were performed on the chromatography scanner. The regions of interest for the three different methods were then applied to the blank scans and the counts in the different regions were recorded for each method. The mean and standard deviation of the 20 sets of counts recorded after scanning was then calculated for each of the three methods.

### *Acceptance criteria*

The LOD was determined by calculating the mean of the counts measured from the blank scans plus three times the standard deviation.

The LOQ was three times the LOD.

### **Robustness**

Radiochemical purity analysis was conducted in two sets. For one set of tests, after placing the spot on the paper strip, it was immediately placed in the development chamber. For another set of tests, after the drop was spotted on a strip, it was allowed to stand for 2 minutes before the strip was placed in the development chamber. The average and the percentage relative standard deviation (% RSD) were determined for each set of results. Also, to compare the two groups Student's T-test for independent samples was used.

### *Acceptance criteria*

Percentage relative standard deviation (% RSD) had to be 15 % or less and for Student T-tests for independent samples,  $p < 0.05$ .

### Data collection and data analysis

The data collected was recorded in a MS Excel spreadsheet and analysed using the STATISTICA. A significance level of 5 % was applied to all the analyses of the validation characteristics that were used in the study. For specificity, accuracy, intermediate precision, linearity within the selected range and robustness, results were presented graphically with the use of means, standard deviations and 95% confidence intervals to describe the estimation for the data collected was not normally distributed.

Specificity was determined firstly by visually identifying the peak for each radiochemical component that was individually tested on the chromatography strips. Secondly, specificity was determined by observing if the calculated concentrations of samples containing varying purities of Tc-99m sestamibi that were tested on the strips corresponded to the recorded percentages of the activity in the regions of interests. Accuracy was determined from the

difference between the mean observed concentration value and the nominal or calculated concentration value. Relative standard deviation was used to report repeatability and intermediate precision while Student's T-test for independent samples was used to compare the different groups for intermediate precision and robustness. For linearity within the selected range, linear regression analysis was used. The standard deviation of the mean counts from blank samples was used to determine the limit of detection (LOD) and the limit of quantitation (LOQ). LOD was three times the standard deviation (3SD) while the LOQ was three times the LOD (3LOD).

## CHAPTER FOUR

### RESULTS

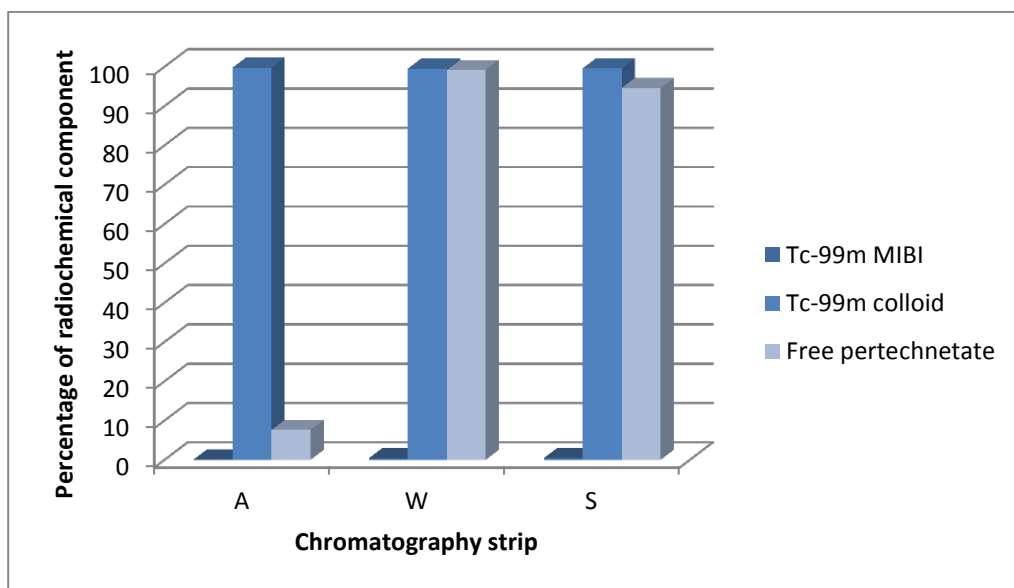
In this study, eight parameters namely specificity, accuracy, precision (repeatability and intermediate), linearity and range, limit of detection, limit of quantitation and robustness were tested during the validation exercise. Each sample that was prepared was tested on the MN Alox (A), Schleicher and Schuell (S) as well as Whatman 31ET (W) RCP analytical methods and results are shown below. A limited number of analyses was performed using Baker-Flex aluminium oxide chromatography strips in order to compare the area of localization of the pertechnetate with results of the Macherey-Nagel (MN) Alox strips. Full details of all the results are provided in Addendum A.

#### SPECIFICITY

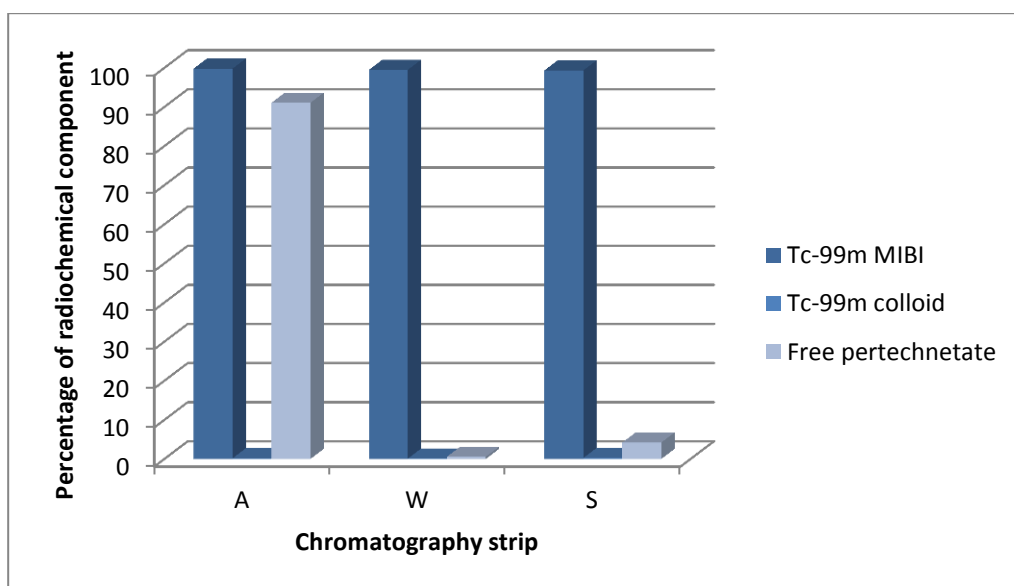
Results where Tc-99m sestamibi, Tc-99m colloid and pertechnetate that were each individually tested on the three chromatography media in order to identify the exact area on the strip where the peak for each radiochemical component would appear are outlined in the graphs below (Figure 1 and Figure 2).

Observation of chromatogram results for samples containing 80.8% Tc-99m sestamibi and 19.2% impurity (mixture of pertechnetate and colloid) revealed two distinct peaks on chromatograms for the MN Alox, Whatman 31ET as well as the Schleicher and Schuell strips in the regions where the impurities and Tc-99m sestamibi were expected to localize (Figure 3, Figure 9 and Figure 10). However, results shown in the graph in figure 2 and the chromatogram in figure 5 show that the peak of pure pertechnetate partly overlaps the area of the Tc-99m sestamibi peak on the MN Alox strip. Drying the strip for a longer period showed a lower  $R_f$  value for pertechnetate but the peak still falls in the region of the sestamibi peak (Figure 6). Pertechnetate on Baker-Flex localized in the designated region for impurities as shown in Figure 8. Separate spotting of Tc-99m sestamibi and pertechnetate showed two peaks that were very close to one another on the MN Alox TLC strip (Figure 4), while on the Baker-Flex TLC strip, the two peaks were well separated (Figure 7).

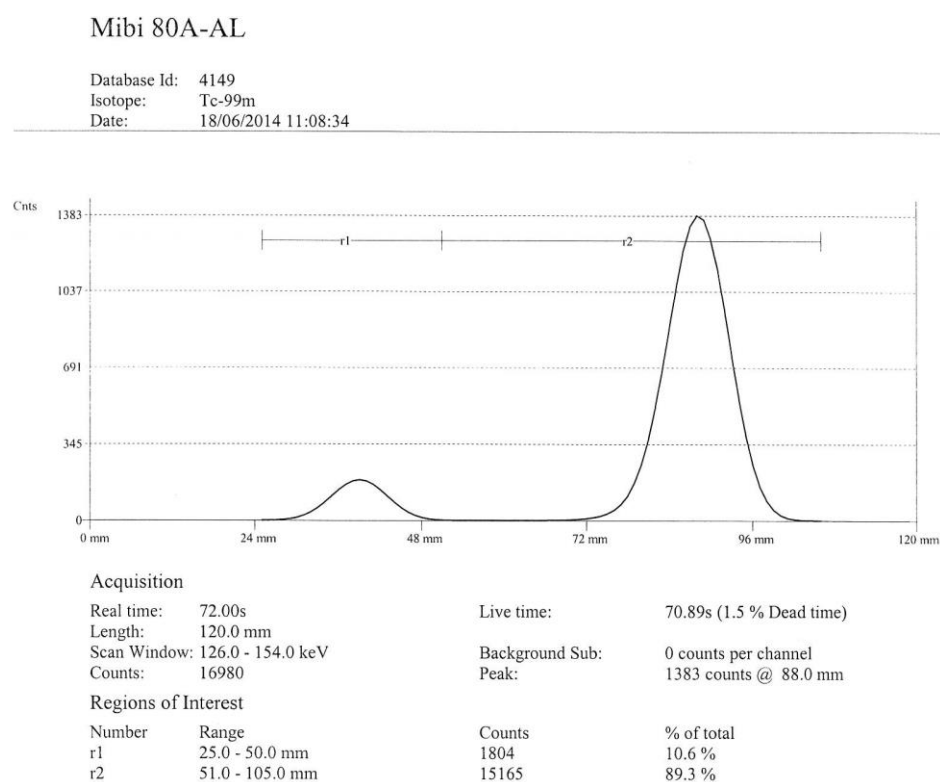


**Figure 1: Percentage of the radiochemical components in the lower ROI**

(A = MN Alox, W= Whatman 31ET, S= Schleicher and Schuell)

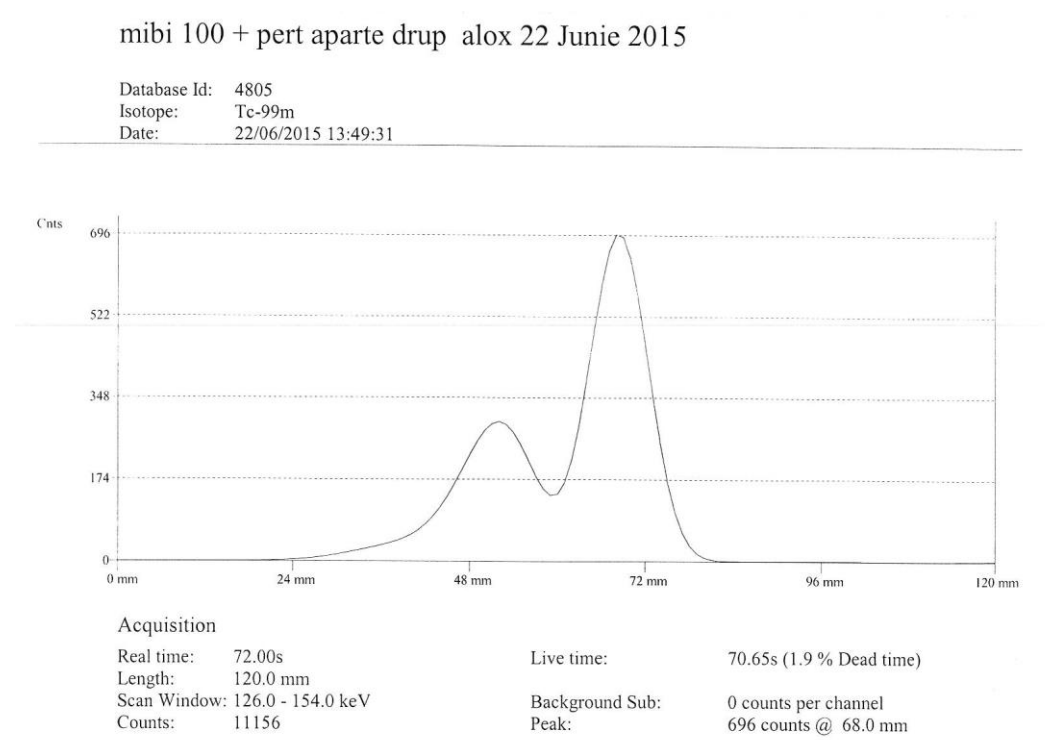
**Figure 2: Percentage of the radiochemical components in the upper ROI**

**Figure 3: Typical MN Alox chromatogram (mixture of Tc-99m sestamibi, Tc-99m colloid and Tc-99m pertechnetate)**

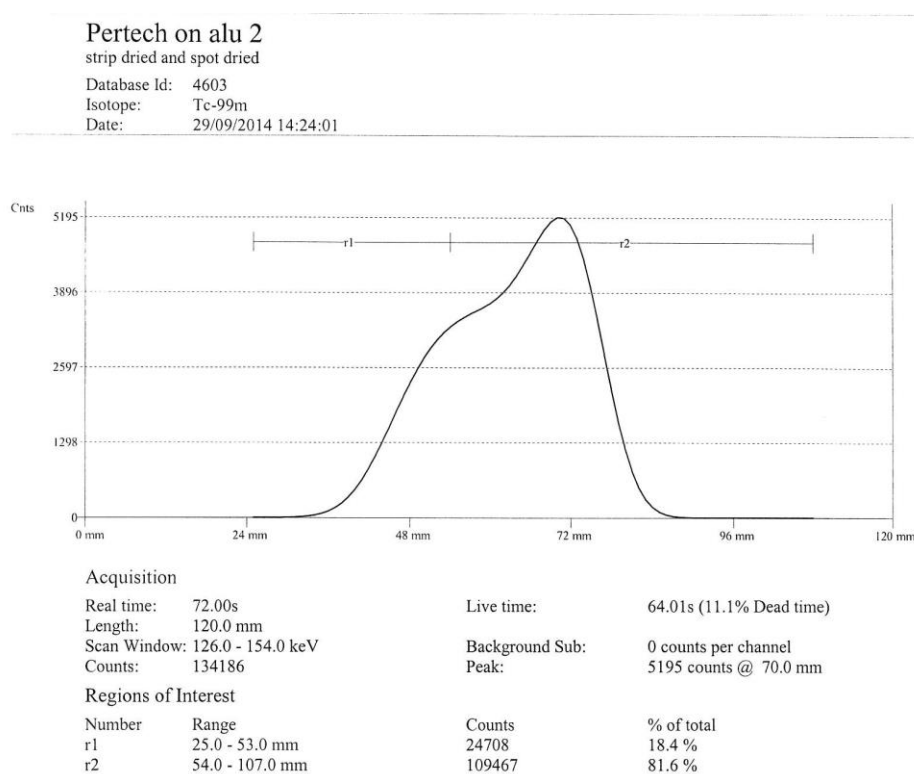


The small peak on the left represents the impurities, while the large peak on the right represents Tc-99m sestamibi. A region of interest (r1) was selected between 25 and 50 mm to determine the counts from impurities, while the region for Tc-99m sestamibi (r2) was selected between 51 and 105 mm. (Note that these distances are measured on the scanner plate, while the strip was shorter. The strip was positioned with its lower edge at the 25 mm mark on the scanner plate.)

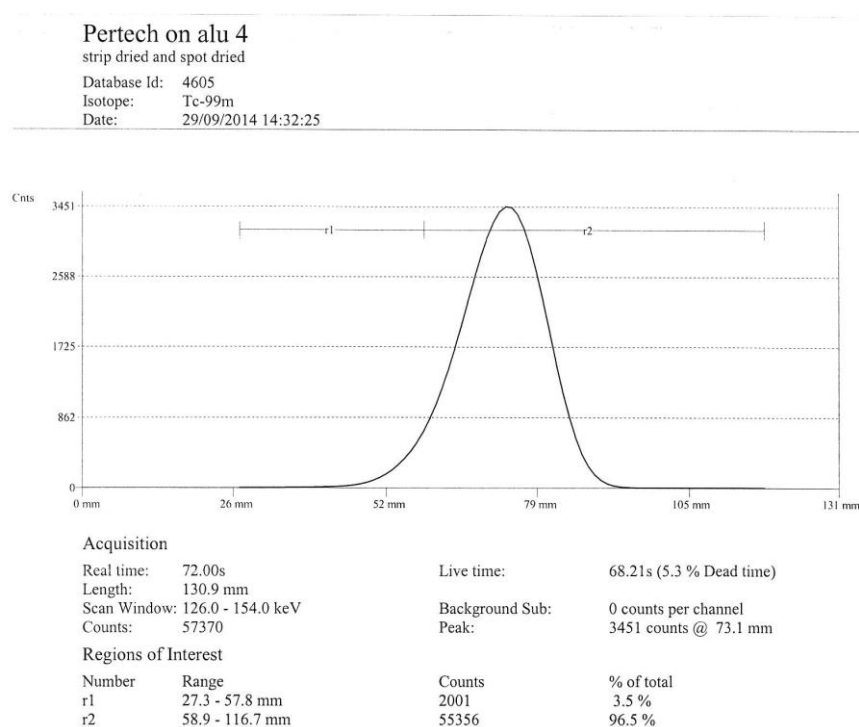
**Figure 4: MN Alox chromatogram (separate spotting of Tc-99m sestamibi and pertechnetate)**



In this case the peak for pertechnetate on the left overlaps that for sestamibi on the right. It is thus not possible to select regions representing the compounds separately, and the counts from the two components cannot be quantified.

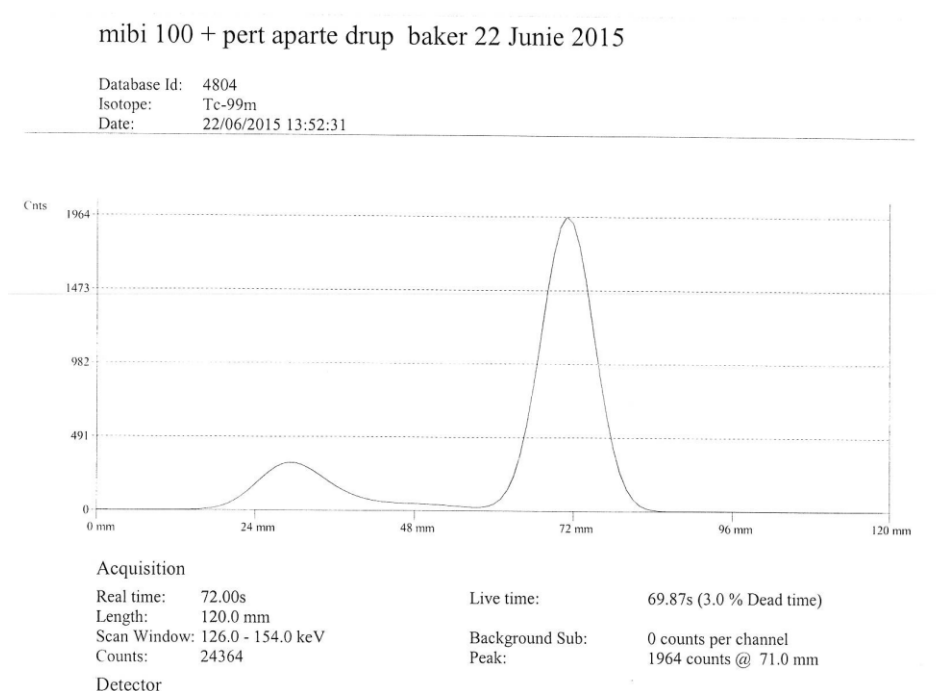
**Figure 5: MN Alox chromatogram (pertechnetate)**

When spotted with pertechnetate only, the area of the peak appears over part of the top half first ROI (designated for impurities) and the lower half of the second ROI (MIBI) of the MN Alox strip. With the pre-set ROIs in the equipment's database for the MN Alox strip, this provides erroneous values for pertechnetate.

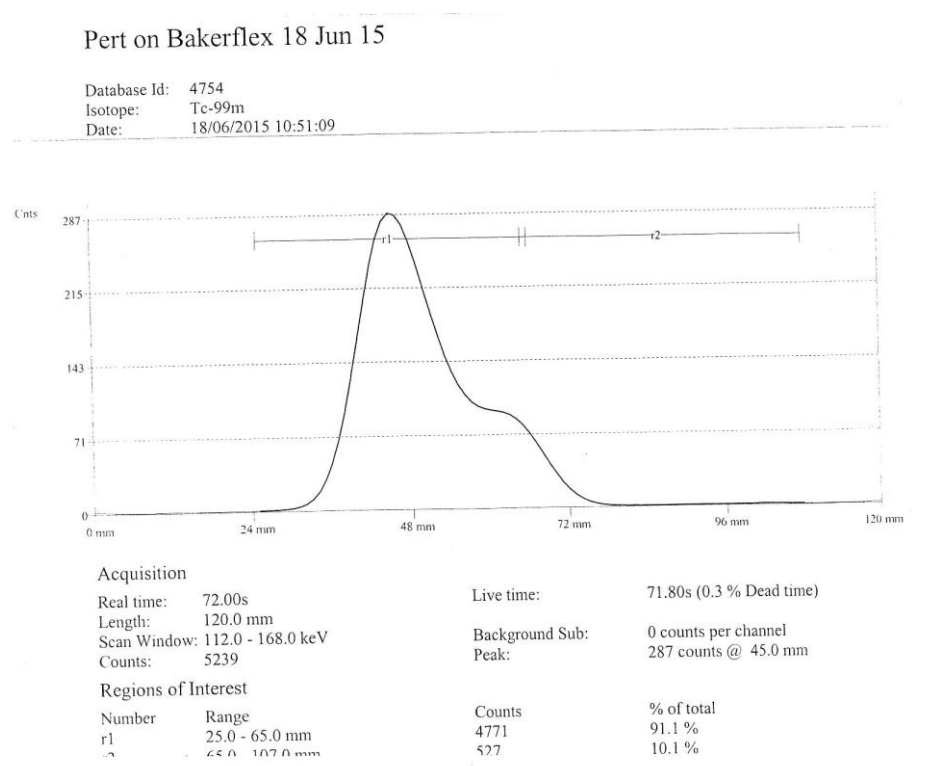
**Figure 6: MN Alox chromatogram (pertechnetate after long drying period)**

Prolonged drying of the MN Alox strip did not improve the result as a very large percentage (96.5 %) of pertechnetate peak appeared in the MIBI ROI on the MN Alox strip.

**Figure 7: Baker-Flex chromatogram (separate drops of Tc-99m sestamibi and pertechnetate)**

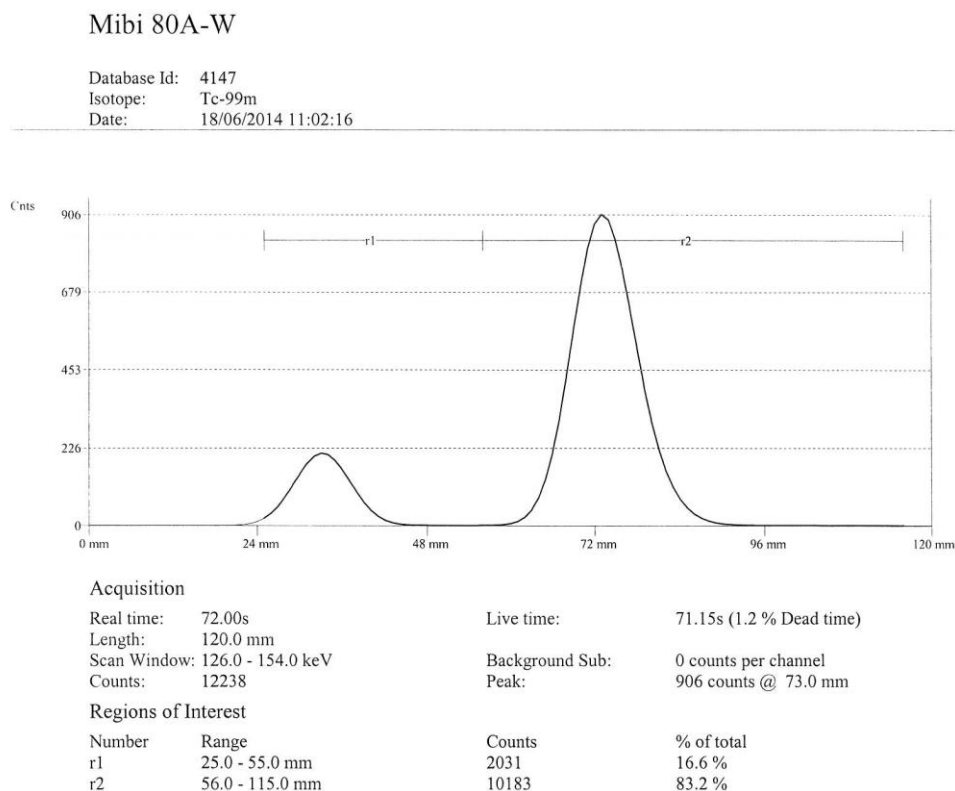


When Tc-99m sestamibi and pertechnetate were both spotted on the same Baker-Flex strip, two peaks that were well separated appeared with the larger peak (Tc-99m sestamibi) appearing in the MIBI region while the smaller peak (pertechnetate) appears in the region designated for impurities.

**Figure 8: Baker-Flex chromatogram (pertechnetate)**

When spotted with pertechnetate only, the peak appears in the region designated for impurities with a very small area of the peak appearing in the MIBI ROI on the Baker-Flex chromatography strip. It is easier to quantify the amount of pertechnetate present with 91.1 % of the impurity appearing in the impurity ROI while a smaller percentage (10.1 %) appearing in the MIBI ROI.

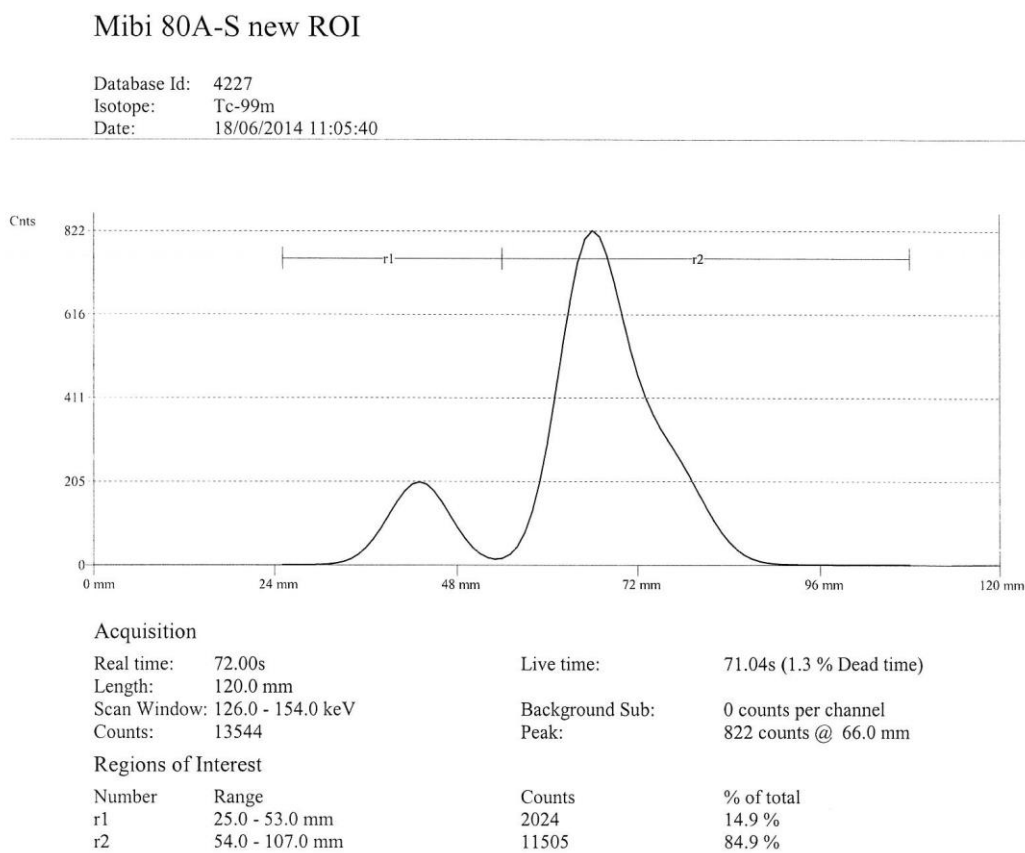
**Figure 9: Whatman 31ET chromatogram (mixture of Tc-99m sestamibi, Tc-99m colloid and pertechnetate)**



Two peaks appeared in the two regions of interest (r1 and r2) when a sample containing a mixture of all three radiochemical components was spotted on the strip. The peaks were well separated making it easier to quantify the peaks in each ROI. The shape of the peak for Tc-99m sestamibi on most of the chromatograms was slightly wider on the Whatman 31ET (Figure 9) when compared to the MN Alox strips (Figure 3).

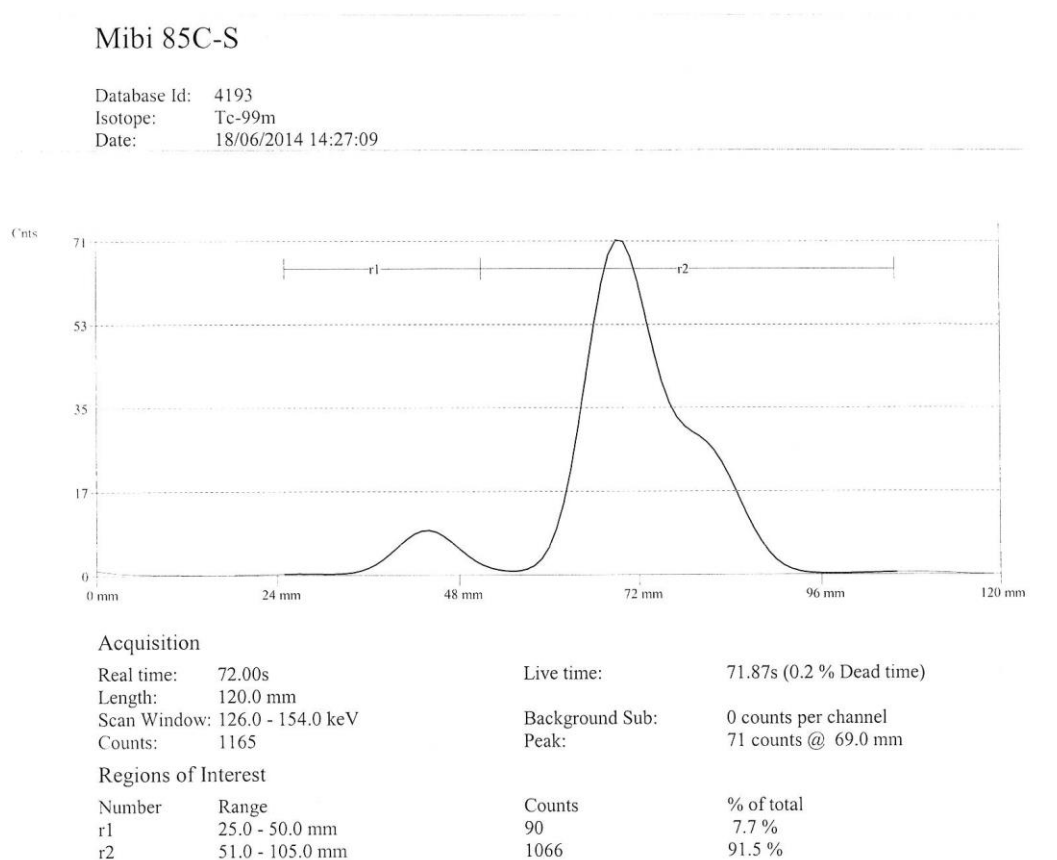


**Figure 10: Schleicher and Schuell chromatogram (mixture of Tc-99m sestamibi, Tc-99m colloid and pertechnetate)**



Two peaks though relatively close, appeared in the regions designated for impurities and MIBI (r1 and r2 respectively) on the Schleicher and Schuell strip. When compared to MN Alox and Whatman 31ET chromatograms, the peaks were narrower and much closer together.

**Figure 11: Schleicher and Schuell chromatogram variant (mixture of Tc-99m sestamibi, Tc-99m colloid and pertechnetate)**



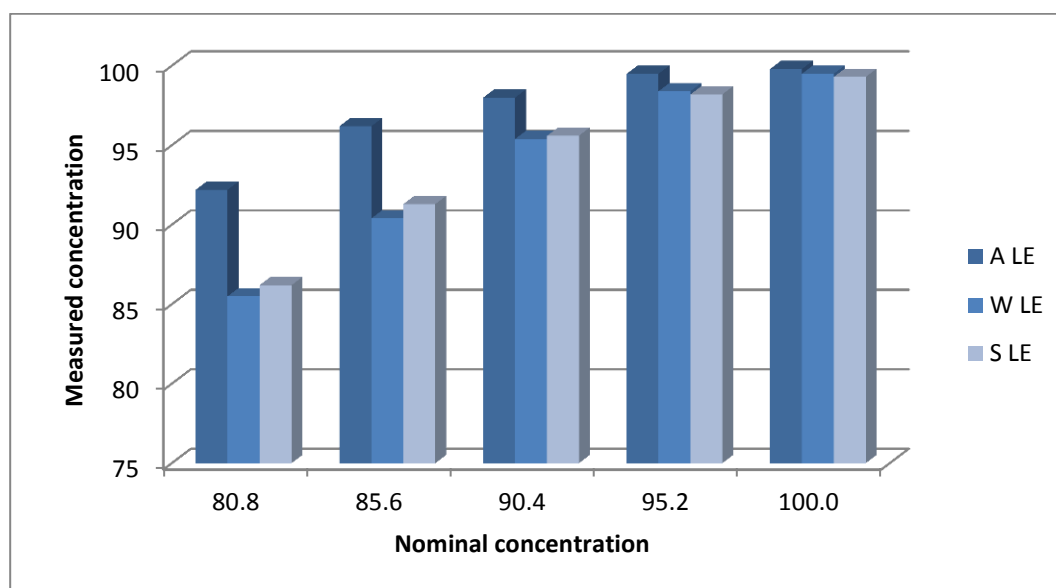
A few Schleicher and Schuell chromatograms also showed very wide peaks in the ROI designated for Tc-99m sestamibi, appearing as though two compounds eluted shortly after each other (Figure 11). However, as both apparent peaks fall into the region designated as MIBI, results from these scans did not differ from the rest of the collected data.

## ACCURACY

The degree of deviation of the measured values from the nominal or calculated values was assessed to determine how accurate each analytical procedure was. For all three RCP test methods, the agreement between the nominal and measured values (Table A<sub>3</sub>) was poor for the samples with high concentrations of added impurities and improved as the impurity concentration decreased. The mean values are shown in Table 1 and Figure 12. This resulted in an overestimation of the RCP results with increasing percentage deviation values as the concentration of the impurities was increased (Table 1). The Whatman 31ET and Schleicher and Schuell methods had fairly comparable mean labeling efficiencies for all the five concentrations. The MN Alox method deviated more from the nominal concentrations. With the acceptance criteria of about 98 % - 102 % of the nominal concentration, only results for 100 % MIBI were within range.

**Table 3: Results indicating the measure of agreement between the nominal (calculated) and the measured concentration (n=4)**

Nominal concentration	MN Alox		Whatman 31ET		Schleicher and Schuell	
	Mean LE (%)	Concentration deviation (%)	Mean LE (%)	Concentration deviation (%)	Mean LE (%)	Concentration deviation (%)
<b>80.8 % MIBI</b>	92.18	+14.10	85.70	+6.06	86.23	+6.71
<b>85.6 % MIBI</b>	96.18	+12.40	90.35	+5.54	91.33	+6.69
<b>90.4 % MIBI</b>	98.00	+8.40	95.40	+5.53	95.63	+5.78
<b>95.2 % MIBI</b>	99.48	+4.50	98.35	+3.30	98.20	+3.15
<b>100 % MIBI</b>	99.80	-0.20	99.50	-0.52	99.34	-0.67

**Figure 12: Comparison between nominal and measured values for Tc-99m sestamibi**

## PRECISION

### Repeatability

Average labeling efficiencies of  $99.8\% \pm 0.1$ ,  $98.2\% \pm 2.2$  and  $99.0\% \pm 0.7$  were obtained for MN aluminium oxide, Whatman 31ET and Schleicher and Schuell paper test methods respectively. All three analytical procedures able to meet the acceptance criteria of  $\% \text{RSD} \leq 15\%$  that was proposed for the study and there was no significant difference for each method (Table 2).

**Table 4: Results of repeatability (n = 8)**

Variable	Valid N	Mean	SD	%RSD
A LE	8	99.78	0.07	0.0709
W LE	8	98.28	2.19	2.2325
S LE	8	98.95	0.66	0.6704

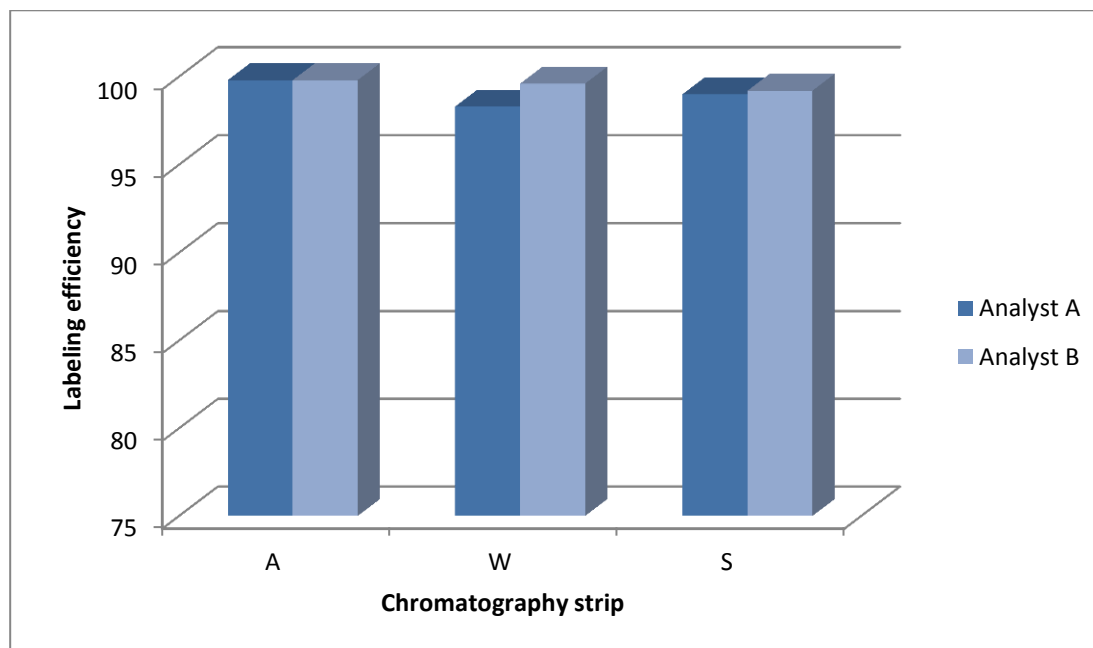
### Intermediate precision

Comparing the three RCP analytical methods using the Student's T- test for independent samples (Table 3 and Figure 13) performed by two different operators (A and B) revealed no significant difference for all three analytical methods indicating that precision is maintained.

**Table 5: Results of intermediate precision for two operators (A and B) (n = 8)**

	<b>A</b>	<b>B</b>	<b>P</b>
	<b>Mean <math>\pm</math> SD (%)</b>	<b>Mean <math>\pm</math> SD (%)</b>	
<b>A LE</b>	99.78 $\pm$ 0.07	99.76 $\pm$ 0.05	0.6927
<b>W LE</b>	98.28 $\pm$ 2.19	99.61 $\pm$ 0.15	0.1074
<b>S LE</b>	98.95 $\pm$ 0.66	99.18 $\pm$ 0.35	0.4091

**Figure 13: Inter-personnel variation**

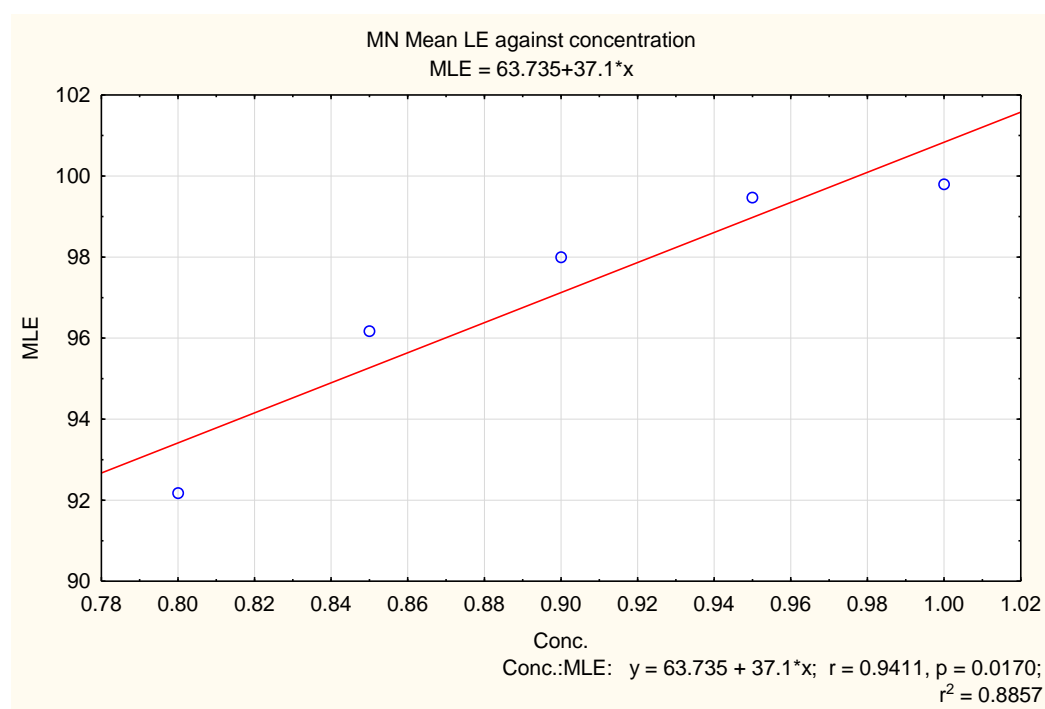


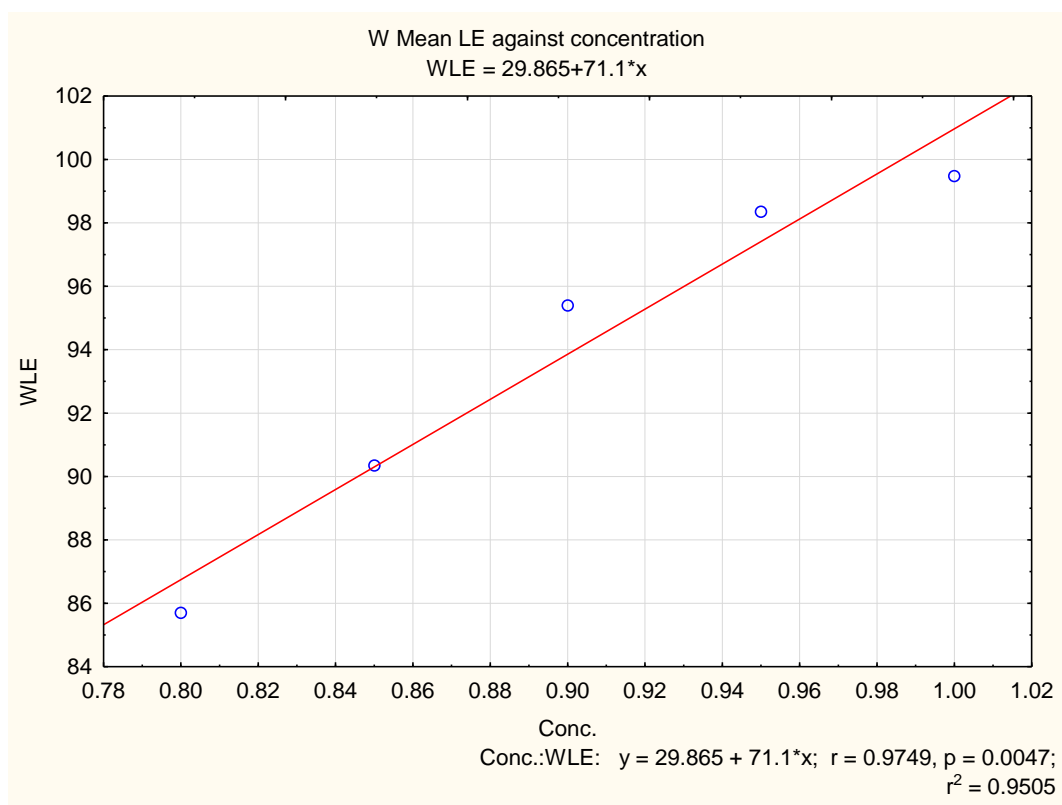
### LINEARITY AND RANGE

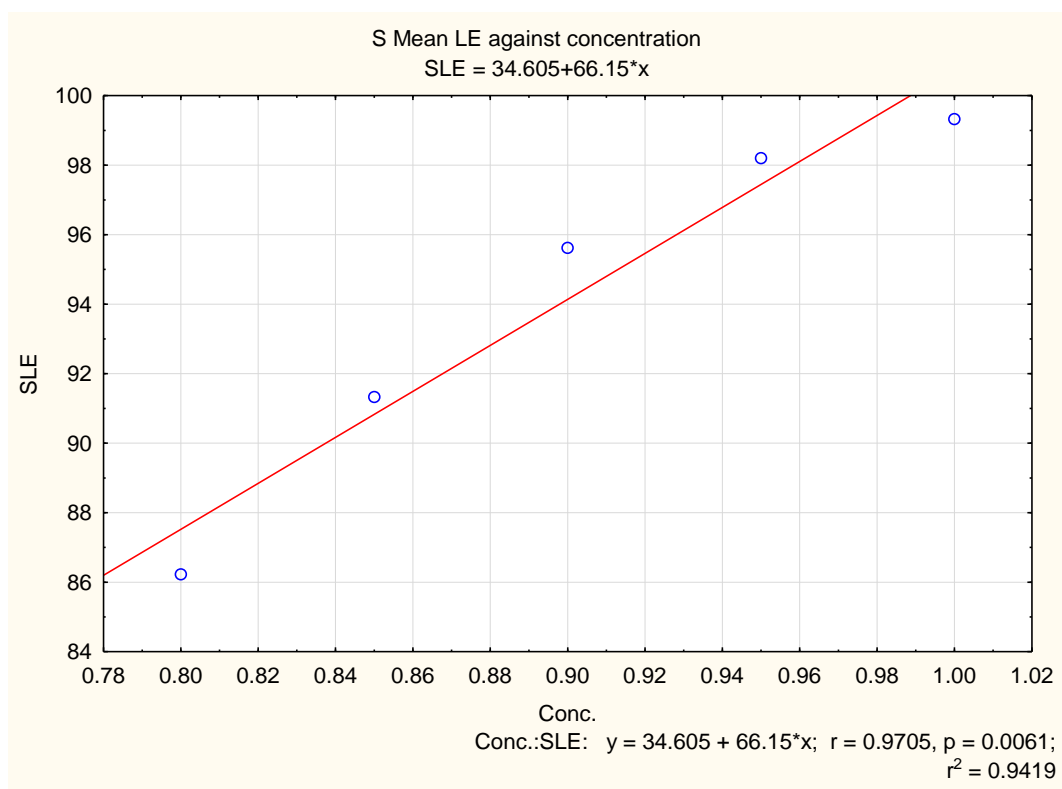
In order to determine the linearity, each RCP analytical method was analysed individually. The relationship between the actual value and the measurement result was found to be positive and significant for three analytical procedures ( $r = 0.9411$ ,  $p = 0.0170$  for MN Alox,  $r = 0.9749$ ,  $p = 0.0047$  for Whatman 31ET and  $r = 0.9705$ ,  $p = 0.0061$  for Schleicher and Schuell).

All three RCP analytical procedures did not meet the proposed limits. The correlation coefficient ( $r$ ) was lower than 0.98, and the Y-intercept was higher than the target in all cases. Results are shown in Figures 14A to C.

**Figure 14A: Results for linearity for MN Alox method**



**Figure 14B: Results for linearity for Whatman 31ET method**

**Figure 14C: Results for linearity for Schleicher and Schuell method**



## LIMIT OF DETECTION AND LIMIT OF QUANTITATION

Determination of detection and quantitation limits was based on the standard deviation of blank samples. A total of twenty blank scans were performed in parallel measurements for all three RCP test methods. The limit of detection (LOD) is regarded as the mean counts of a blank scan plus 3 standard deviations of the mean. The limit of quantitation (LOQ) is three times the LOD. Results in Table 4 show that for the radiochromatogram scanner that was used for this study, over the 120 mm scan length, 59 counts and 177 counts were determined as the limit of detection and limit of quantitation respectively. Variations in the number of counts in the specific ROIs were due to differences in the lengths of the regions designated for either the compound of interest or the impurities for the three different analytical methods.

**Table 6: Results of analysis of blank scans (n=20)**

	Total cts			Total cts			Total cts		
	W1	W2	W	S1	S2	S	AL1	AL2	AL
Mean	12.35	20.25	38.50	9.50	17.90	38.50	10.45	16.95	38.50
SD	4.32	4.59	6.85	3.33	3.90	6.85	3.63	3.83	6.85
3SD	12.96	13.76	20.54	10.00	11.80	20.54	10.90	11.49	20.54
LOD	25.31	34.01	59.04	19.50	29.70	59.04	21.35	28.44	59.04
LOQ	75.93	102.03	177.12	58.50	89.10	177.12	64.05	85.32	177.12

cts= counts

W1 = Whatman 31ET region 1, W2 = Whatman 31ET region 2, W = Whatman 31ET region 1 and region 2

S1= Schleicher and Schuell region 1, S2= Schleicher and Schuell region 2, S = Schleicher and Schuell region 2 and 2

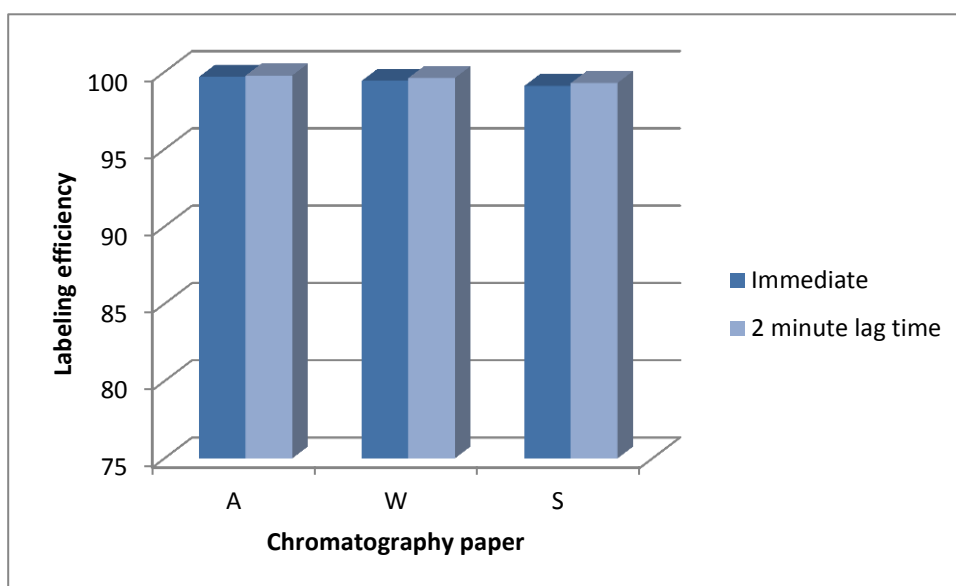
AL1 = MN Alox region 1, AL 2 = MN Alox region 2, AL = MN Alox region 1 and 2

## ROBUSTNESS (Lag time between spotting and strip development)

Results in Table 5 show labeling efficiencies for samples without (group 1) and with (group 2) a two minute lag time indicate that the effect of two minute lag time between spotting and placing the strip in the chamber. The lag time did not affect the RCP results of all three analytical methods. There was no significant difference between the two groups indicating that the RCP analytical methods were robust (Figure 15).

**Table 7: Results of robustness****Comparison with (group 1) and without (group 2) two minute lag time (n = 15)**

	<b>1</b> Mean $\pm$ SD (%)	<b>2</b> Mean $\pm$ SD(%)	<b>P</b>
<b>A LE</b>	99.77 $\pm$ 0.06	99.71 $\pm$ 0.10	0.0679
<b>W LE</b>	98.94 $\pm$ 1.65	99.35 $\pm$ 0.47	0.3631
<b>S LE</b>	99.06 $\pm$ 0.52	99.18 $\pm$ 0.44	0.5056

**Figure 15: Comparison between the effect of immediate and 2 minute lag time**

## **CHAPTER FIVE**

### **DISCUSSION AND CONCLUSION**

#### **DISCUSSION**

In the pharmaceutical manufacturing industry, which includes radiopharmaceutical manufacture, strict adherence to GMP principles <sup>[1]</sup> is required to ensure that any medicine that is produced is safe and effective <sup>[1]</sup>. Analytical procedures are one example of quality control tests that are used by the manufacturer to determine the purity of a medicine. They achieve this by identifying and quantifying the compound of interest and any impurity/s that might have been formed thereby ensuring that the correct specifications of the products are met during the manufacturing process <sup>[5]</sup>. Any analytical procedure employed should therefore be validated in order to make it more reliable and increase the credibility of the test results <sup>[1-5, 7-10]</sup>. In Nuclear Medicine, the use of validated analytical procedures in determining the RCP of radiopharmaceuticals helps to ensure that only products of acceptable quality are administered to the patient <sup>[17, 19]</sup>.

Validated methods are described in pharmacopoeias as well as in package inserts or product specifications of radiopharmaceuticals provided by manufacturers <sup>[12, 22-27]</sup>. However, the prescribed analytical procedures tend to be slow or too complicated for the user who needs to use an analytical procedure after labeling the kits with Tc-99m in a hospital radiopharmacy. Many radiopharmacies, including those in Africa, have incorporated simpler and more practical alternative RCP analytical methods, which may not have undergone a full analytical method validation. Regulatory guidelines strongly recommend that any facility that intends to implement an alternative analytical procedure validates the new test method <sup>[2-5, 11-14]</sup>, but there are limited guidelines on validation of RCP analytical methods and also, many hospital radiopharmacies are without good analytical facilities and equipment to undertake such an exercise.

In order to determine whether a RCP test method can be validated in a hospital radiopharmacy setting in Southern Africa, this study attempted to undertake a full method validation of two alternative analytical procedures based on ICH Q2A and Q2B guidelines <sup>[6, 14]</sup> versus a manufacturer's method and to establish a template for methods validation. The study concentrated on alternative methods used in RCP analysis for Tc-99m sestamibi.

Each radiopharmaceutical is supplied with a package insert in which the manufacturer recommends a specific analytical method to be used and provides information on the type of materials needed and step by step instructions on how to perform the analytical test <sup>[12, 22-27]</sup>. For Tc-99m sestamibi, Baker-Flex aluminium oxide-coated plastic thin layer chromatography strips as stationary phase and ethanol ( $\geq 95\%$ ) as the mobile phase are recommended <sup>[25, 26]</sup>. This study therefore compared Whatman 31ET <sup>[33]</sup> (used at Tygerberg Hospital) as well as Schleicher and Schuell (used at Groote Schuur Hospital) as stationary phases with 0.20 mm aluminium oxide on pre-coated plastic sheets (POLYGRAM® ALOX N manufactured by Macherey-Nagel in Germany). The Alox TLC strips that could be procured locally were assumed to have properties that were comparable to those of Baker-Flex aluminium oxide strips. The procedure for the MN Alox strips as the stationary phase with ethanol as the mobile phase was based on the procedure described by Lantheus Medical Imaging, the suppliers of the patented Cardiolite sestamibi kit. A small supply of Baker-Flex strips could be obtained at a late stage of this study, allowing only a limited comparison with the other methods.

The two alternative analytical procedures that were used in the study differed from the analytical procedures involving recommended Baker-Flex and substitute Macherey-Nagel (MN) aluminium oxide regarding preparation conditions and step by step procedure. The recommended and the MN Alox analytical procedures were both observed to be time consuming, having a developing time of between 16 and 23 minutes. With the need to use Tc-99m sestamibi in some emergency procedures as well as due to the short half-life of Tc-99m, paper chromatography methods have been described which are quicker and required fewer preparation steps <sup>[29-33, 41, 43]</sup>. With a developing time of between 3 to 5 minutes, the paper chromatography methods are preferable and have already been incorporated in a number of hospital radiopharmacies. In the current study, mean labeling efficiencies for sestamibi samples without any added impurities (Tc-99m colloid and pertechnetate) were  $99.8\% \pm 0.0\%$  for Macherey-Nagel Alox TLC,  $99.5\% \pm 0.1\%$  for Whatman 31ET paper and  $99.3\% \pm 0.2\%$  for Schleicher and Schuell paper chromatography strips. However, an overestimation of the observed results (Table A<sub>3</sub>) when compared to the calculated values (Table A<sub>2</sub>) was noted for samples that were prepared by mixing already prepared Tc-99m colloid and pertechnetate to the already prepared and cooled Tc-99m sestamibi. Possible causes of these findings are described below

## Validation Parameters

In an attempt to undertake a full analytical method validation based on ICH Q2A and Q2B guidelines <sup>[6, 14]</sup>, eight validation parameters namely specificity, accuracy, precision (repeatability and intermediate precision), linearity, range, limit of detection, limit of quantitation and robustness were selected for the current study.

For specificity, the RCP analytical method had to demonstrate the ability to separate the Tc-99m sestamibi from other components such as pertechnetate, insoluble hydrolyzed reduced Tc-99m (colloid) and Tc-99m pentamibidimethylvinylisonitrile <sup>[22]</sup> that could have been present in the sample <sup>[16, 19]</sup>. In the pharmaceutical industry, reference standards that are used to identify the compound of interest and impurities expected in a drug product, are available from official sources <sup>[53]</sup>. For the current study however, certified standards of each of the expected radiochemical components in the radiopharmaceutical were not available due to the short half-life of Tc-99m and short shelf-life of the radiolabeled product. Preparation of such standards would require them to be used a few hours after preparation. The standards for pertechnetate and colloid impurities were prepared in house with reasonable certainty that they represented known quantities. For 100 % Tc-99m sestamibi, the radiopharmaceutical was prepared according to the manufacturer's instructions and used shortly afterwards, assuming it to be 100 % pure.

Tc-99m sestamibi, pertechnetate and tin colloid were each individually tested on the MN Alox, Whatman 31ET, and Schleicher and Schuell paper chromatography strips. Our findings revealed that the peaks for the impurities (pertechnetate and colloid) and Tc-99m sestamibi that were each individually tested on the Schleicher and Schuell as well as Whatman 31ET paper chromatographic strips appeared in the expected ROIs (figures 1 and 2). This was due to the poor interaction of the impurities with the non-polar lipophilic solvent, resulting in their retention near the origin of the chromatographic strips<sup>[21]</sup>, while the lipophilic Tc-99m sestamibi moved with the solvent front. Our results for MN Alox TLC strips were contrary to what was expected. Being an anion exchange material, aluminium oxide was expected to retain the pertechnetate near the origin <sup>[21]</sup> but the current study showed that the pertechnetate moved further along the strip with the peak, partially overlapping with that of Tc-99m sestamibi. The poor separation of this impurity from the Tc-99m sestamibi resulted in an overestimation of the sestamibi fraction, and therefore of the radiochemical purity (RCP).

Samples containing varying concentrations of Tc-99m sestamibi, pertechnetate and tin colloid were also tested on the analytical procedures in order to determine whether each RCP test method would be able to separate and correctly quantify the Tc-99m sestamibi and the impurities present. These samples were prepared by adding known quantities of the impurities to the sestamibi vial after cooling. However, an overestimation in the RCP test results was observed for all the RCP test methods. This was most likely due to other chemical forms of technetium that could have been formed and probably co-migrated with the Tc-99m sestamibi. A number of studies in the literature involving the use of Baker-Flex TLC strips have also reported similar findings to the current study that are suggestive of co-migration of some impurities with Tc-99m sestamibi [32, 35, 36]. Proulx et al. [32] attributed the co-migration to inadequate drying of the TLC strips. However, according to our findings, the overestimation of RCP test results had been observed in all the RCP test methods indicating that in our case, the problem was most likely due to the sample and not the analytical procedure. Although adequately drying the MN Alox strips led to a slight improvement in peak position (Figure 6), the pertechnetate could not be completely separated from the Tc-99m sestamibi. The  $R_f$  of pure pertechnetate on the MN Alox TLC strip did indeed fall in the ROI designated for Tc-99m sestamibi (51 to 105 mm from the start of scanning).

Thomson et al [36] identified two lipophilic impurities using HPLC while Cooper et al [35] also explained that the impurities were most likely to be isonitrile complexes. Unfortunately for the current study, neither paper nor TLC methods could separate isonitrile impurities from sestamibi. In the current study it was therefore not possible to test for the isonitrile impurities [22] which limited the efficiency of the analytical methods.

Accuracy can be demonstrated by a number of ways that are described by the ICH guidelines [1, 2, 4, 6-11, 13-15]. Firstly, accuracy can be demonstrated by comparing how close the results of a proposed analytical procedure are to the concentration values of a reference material or sample of known purity. It can also be determined by comparing the results of a proposed analytical procedure to results of an analytical procedure that has already been validated. Finally, accuracy can be concluded in instances where the precision, linearity and specificity of an analytical procedure are established. For radiopharmaceuticals, accuracy has mostly been determined by comparing results obtained from the manufacturer's recommended analytical method which is considered to be the reference method, to the results of the proposed method [29 - 32, 52,]. In the current study however, due to the problem with specificity with the MN Alox TLC test method, it could not be used as a reference method where re-

evaluation of the paper chromatography methods against the prescribed Baker-Flex method should be considered to confirm the accuracy of the faster methods.

To determine accuracy in the current study, samples were first prepared by mixing known quantities of the two impurities with sestamibi solution in a vial and calculating the concentrations of Tc-99m sestamibi, pertechnetate and colloid in the mixture (Table A<sub>2</sub>). The agreement between the calculated and the measured concentrations of the radiopharmaceutical compound was then evaluated. Our results showed that only samples that were prepared according to the manufacturer's prescribed instructions without addition of any impurities (100 % MIBI samples) had good agreement between the theoretical and measured values (Table A<sub>3</sub> and Figure 12). For the lower concentrations, where known amounts of impurities were added, the measured values differed from the theoretical values and therefore the results were not accurate. This poor agreement between the theoretical and observed results could have been due to the formation of other impurities as described above, which none of the RCP test methods was able to separate from the Tc-99m sestamibi. The overestimated values for Whatman 31ET were very close to the values recorded for Schleicher and Schuell while the MN Alox test method recorded higher sestamibi values. For the MN Alox test method, the observed values could have been due to the co-migration of the pertechnetate in addition to other impurities that could have been present in the samples. An analytical procedure could also be said to be accurate once linearity and specificity parameters are established. However, for our study, all three analytical methods did not meet the specificity and linearity due to the poor agreement between the observed and calculated values.

The precision of an analytical procedure is demonstrated when repeated measurements from a homogenous sample closely agree <sup>[1, 2, 4, 6-11, 13-15]</sup>. With the proposed acceptance criteria % RSD of  $\leq 15\%$  <sup>[54]</sup>, all the RCP test methods were able to meet the proposed limit. Also despite variation in the distribution of data obtained from two different operators, our results revealed no significant difference between their results, indicating good intermediate precision for all three analytical procedures as the same acceptance criteria for the intermediate precision was maintained.

Within a selected range, test results of an analytical procedure undergoing validation have to show proportionality to the concentration of the analyte in order to prove linearity <sup>[1, 2, 4, 6-11, 13-15]</sup>. In the current study, the observed results were expected to show proportionality to that of the nominal concentrations that were determined in table A<sub>2</sub>, since Tc-99m sestamibi and Tc-

<sup>99m</sup>Tc colloid were prepared according to the manufacturer's instructions while the free pertechnetate that was obtained from a fresh eluate were all assumed to be almost 100 % pure. Our results instead showed an increase in the observed values as the <sup>99m</sup>Tc sestamibi concentration decreased with y-intercept values that significantly departed from zero. Based on the acceptance criteria for the correlation coefficient ( $r \geq 0.98$  %) <sup>[11]</sup> and the y-intercept of  $\leq 5$  % of the target concentration <sup>[55]</sup> that were proposed for the current study, our results for all three RCP analytical procedures did not meet the limits. Also, Krause attributed the y intercept values which were significantly greater than zero to be due to systematic errors or flaws such as how the sample was prepared. This could be true in that a systematic error could have resulted in the formation of the unknown impurity and it was not known if the impurities were formed during the compounding the individual or after mixing the already formed radiochemical components. Our results ( $r = 0.9411$  for MN Alox method,  $r = 0.9749$  for Whatman 31ET method and  $r = 0.9705$  for Schleicher and Schuell method) for the correlation coefficient were however in close range to those reported by Hung et al. <sup>[30]</sup> and Luebke et al <sup>[31]</sup>, although the acceptance criteria that was used was not mentioned in these studies. Hung et al had  $r$  values of 0.98 and 0.88 with MPC and SPE respectively while Luebke et al <sup>[31]</sup> had  $r$  values of 0.80 and 0.83 for the mini and regular Whatman 31ET when they were all compared to the results of the Baker-Flex TLC analytical method. Huber <sup>[7]</sup> also described that y intercept values that significantly departed from zero could be accepted if the accuracy of the analytical procedure was not affected. For the current study, accuracy could not be established for any of the three RCP test methods due to the poor agreement between the observed and nominal values.

Detection and quantitation limits are parameters that have been used to describe the smallest concentration of an analyte in a sample that can be reliably measured by an analytical procedure <sup>[1, 2, 4, 6-11, 13-15, 56, 57]</sup>. In our case, the parameters are determined for the specific counting equipment used and are only indirectly based on the chemical properties of the radiopharmaceuticals. Low counts tend to be affected by background activity <sup>[51, 56-58]</sup>. Counts that exceed 3 times the background reading should be distinguishable from background <sup>[51, 58]</sup>. This value is therefore used as LOD. In the current study, the limit of detection for the region of interest of 120 mm of the radiochromatogram scanner was approximately 60 counts. These counts are very low and not usually counted on the scanner for radiopharmaceuticals prepared in routine practice. At our institution, sestamibi analysis usually is in the range of 16 000 to 20 000 counts on the entire strip. The lowest range if the kit is prepared for a limited number of



doses, is expected to still exceed 4 000 counts for the strip. Thus, even for the lower of the expected total counts, an impurity peak with 100 counts i.e. 2.5 % of the radioactivity on the strip should be detectable. Detection and quantitation limits have not been viewed as necessary and have not been included in published articles on validation of RCP test methods. Seetheraman et al <sup>[52]</sup> argued that it was because values below the RCP limit were not useful clinically. For Tc-99m sestamibi, the RCP limit is 90 %, impurity concentrations of 10 % and less will not affect the quality of the image. Thus quantitation of impurities smaller than 10 % of the total radioactivity of the product would have no clinical significance. But Armbruster and Pry <sup>[56]</sup> reasoned that the two parameters were in fact essential in that the capability or limitations of the equipment should be known.

Robustness assesses whether the analytical procedure is able to remain unchanged by small but deliberate variations to the procedure during normal usage <sup>[1, 2, 4, 6-11, 13-15]</sup>. In hospital radiopharmacy settings, some of the variations are inevitable; hence it is important to know how reliable the analytical procedure is when such variations occur. The current literature provides examples of variations that could be assessed for HPLC<sup>[1-4, 6, 11, 12]</sup>, but none have provided for other chromatographic methods such as paper or TLC chromatography. For the current study, the effect of lag time between spotting and placing the strip in the chamber was assessed since the hospitals where the alternative methods are used are teaching hospitals. Students who are also assigned to compound and perform RCP tests may work slowly due to lack of experience. Results showed that the effect of 2 minute lag time did not affect RCP results of all three analytical methods indicating that they were robust. Examples of other variations such as drop size when spotting, lag time, how the mobile phase and stationary phase are stored as well as the type of developing chamber could be looked into in future validation processes.

### **Summary of the validation study**

In summary, the findings of the current study have shown that all eight validation characteristics are important and should be included when validating the RCP test methods. In other similar studies, limit of detection and limit of quantitation parameters were not included as they were noted to be difficult to determine <sup>[51]</sup>. However, with the variation in the sensitivity to the low counts of the different types of radiation detecting equipment used in radiopharmacy, LOD and LOQ determination for the equipment were noted to be in fact important. Results of the number of counts obtained from blank samples would enable the

analyst to know the average counts that are detected as background radiation and this would guide how much activity could be added to a chromatography strip in order to provide an effective reading without background interference.

For some parameters, namely specificity, accuracy and linearity, the RCP test methods were not able to meet the acceptance criteria that were set for the current study. All the three analytical procedures overestimated RCP test results, possibly due to the formation of another form of technetium compound which co-migrated with the Tc-99m sestamibi fraction and hence could not be isolated by the analytical methods. It could not be proved that this impurity was Tc-99m pentamibi and it was not possible to prepare a standard for this impurity in-house. As a result, this limited the credibility of our results regarding specificity. The overestimated values for the Whatman 31ET and Schleicher and Schuell analytical methods were quite similar while for MN Alox, the higher values that were recorded were most likely due to co-elution of the free pertechnetate in addition to the unknown impurity. As a result, the MN Alox RCP test method could not be used as a reference standard. The poor agreement between the nominal and observed results had a negative effect on the accuracy and linearity of the analytical procedures. Apart from meeting the acceptance criteria for repeatability and intermediate precision, all three methods were also noted to be robust.

### **Guidelines and challenges**

The current guidelines on validation of analytical procedures that are applicable to general medicines in the manufacturing industry have been provided by the International Conference for Harmonization (ICH) <sup>[4, 12]</sup>, international regulatory bodies and other published literature <sup>[12, 22]</sup>. They have been used as reference points by many national authorities for example the FDA in the USA <sup>[14]</sup>. RCP analysis is part of quality control of radiopharmaceuticals which assures safety, efficacy and quality. Radiopharmaceuticals are medicines hence subject to pharmaceutical regulation <sup>[16, 19, 47]</sup>. The ICH guidelines are applicable to manufacturers of radiopharmaceuticals. In small scale preparation such as in hospital radiopharmacy however, guidelines for manufacturers may not be relevant. Limited guidelines are available on how to validate RCP test methods. Elsinga et al <sup>[46]</sup> briefly described the parameters namely repeatability, linearity and specificity that could be used to validate RCP test methods but did not provide detailed information. Many published articles on RCP analytical method validation have shown slight variations in the number of parameters used with some comparing only accuracy, linearity and specificity <sup>[29-31]</sup> while other articles tried to validate

using all eight parameters<sup>[51, 52]</sup>. Leonardi et al<sup>[51]</sup> noted that LOD and LOQ were difficult to determine since they were dependent on the intrinsic characteristics of the radiation detecting equipment where data at low counts were largely affected by background radiation and therefore could not provide reliable results.

## Limitations

This study has a number of limitations. Availability of certified radiopharmaceutical standards is hampered by the short half-life of Tc-99m. There were no certified standards available from official sources and hence pertechnetate and tin colloid were added to the kit to represent the impurities that could result in poor labeling. It was assumed that strict adherence to the method of preparation of each component would result in an almost pure product<sup>[16-22]</sup>. It was also not possible to identify all the impurities with the analytical procedures that were used for the study. Tc-99m pentamibidimethylvinylisonitrile<sup>[22]</sup> is one of the potential impurities of Tc-99m sestamibi but unfortunately this radiochemical component could not be prepared in house or isolated by the analytical procedures that we used for the study.

The use of HPLC would have provided very useful information on isonitrile impurities that may have been present but could not be separated from the Tc-99m sestamibi by TLC or paper chromatography. This is because of the high resolution and better distinction between the different substances<sup>[15-21]</sup>. An HPLC was however not available at the study site.

The prescribed Baker-Flex aluminium oxide plates could initially not be sourced in South Africa. An alternative similar TLC strip that was available locally was therefore included in the study. However, this generic aluminium oxide overestimated the sestamibi fraction because of poor separation between sestamibi and pertechnetate.

Some of identified shortfalls did have an effect on the credibility of the results. The alternative analytical procedures could not be effectively compared to the recommended method due to non-availability of specific materials required for the reference analytical method. The material (MN Alox chromatography strips) that was used did not provide accurate results due to overestimation of the RCP. For the validation process, it is important that the materials specified by the manufacturer are used. Availability of HPLC would help provide more accurate information concerning not only the impurities that are not identified or separated from the Tc-99m sestamibi, but can also be used to check how pure the in-house prepared standards are.

## Recommendations

Based on the findings of the current study, a template protocol for validation of RCP test methods is proposed (refer Addendum B). The validation protocol or a validation masterplan which contains all the appropriate information and step by step instructions has to be available before the validation exercise can be carried out. A format for the contents of validation protocols used in the pharmaceutical industry has been provided by a number of published papers and regulatory guidelines and the basic contents required include the objective and scope, the validation parameters to be assessed and acceptance criteria as well as the type of validation experiments to be performed<sup>[1, 2, 7, 8, 10]</sup>. For this study, this method validation protocol has been adapted specifically for RCP analysis by TLC, ITLC or paper chromatography.

A successful validation exercise requires joint effort from key personnel attached to the facility that intends to implement a new analytical method. Their roles and responsibilities have to be identified and clearly indicated in the protocol<sup>[1, 2, 7, 8, 10]</sup>. In a hospital radiopharmacy, apart from the analyst who would take a great deal of time to prepare the protocol and conduct the validation experiments, other nuclear medicine personnel, for example the nuclear medicine physician or the medical physicist, can also assist with editing and/or approving the protocol.

The ICH Q2A and Q2B guidelines<sup>[6, 11, 14]</sup> used in pharmaceutical manufacturing industry have provided general guidelines on how to categorize an analytical procedure and how to select up to eight validation parameters to be used to assess an analytical procedure. For the current study, the RCP test methods were categorized in all the four assay groups described by the ICH Q2A guidelines to which all the eight validation parameters were required. The categorization was based on the ability of the RCP analytical procedure to identify and quantify the analyte and impurities<sup>[6, 11]</sup>. However, a number of published articles on validation of RCP test methods have not included detection and quantitation limits. Properties of radiation detecting equipment<sup>[46, 51]</sup> and actual counts used in routine practice<sup>[52]</sup> are far above the detection and quantitation limits and have attributed to them not being included but their determination would help the analyst ensure that sufficient radioactivity is used.

The specific design of validation experiments for radiopharmaceuticals, like ordinary medicines, rests on the analyst intending to validate the analytical method. However, the analyst has to conform to the basic information on experimental design and statistical analysis

provided in the ICH Q2B guidelines<sup>[14]</sup>. For the current study, the basic experimental design was similar to those of a number of other published articles on RCP test method validation<sup>[29-32, 41, 42]</sup>. ICH guidelines however do not outline exact values or limits and do not provide information on how to derive the acceptance criteria. This could be challenge in some hospital radiopharmacies with personnel who have not received specialist training, since deriving the acceptance criteria requires some form of expertise. If not correctly derived, it can result in a failed process with the need to repeat the validation exercise<sup>[11]</sup>.

In the pharmaceutical industry, there are some specific values or limits that have generally been used as acceptance criteria for a number of validation parameters in analytical method validation. For the current study, the acceptance criteria of 98 % to 102 % of the nominal (calculated) concentration was selected to report how accurate the RCP test methods were and is similar to the acceptance criteria that is reported for percentage recovery<sup>[1, 59]</sup>. For repeatability and intermediate precision, the % RSD  $\leq$  15 % described by Arieta<sup>[54]</sup> was proposed for the study. Another value for the % RSD  $\leq$  10 % had been described by Zigler<sup>[50]</sup> as the acceptance criteria for repeatability for test methods for PET radiopharmaceuticals namely [<sup>18</sup>F]-FDG. This value however has been derived using acetonitrile which is used in the synthesis of [<sup>18</sup>F]-FDG and is present in the final product as a chemical impurity. Variations on how linearity has been reported has been noted in a number of published literature with Hung et al<sup>[29,30]</sup> and Luebke<sup>[31]</sup> reporting only the correlation coefficient (r) while Seetharaman et al.<sup>[52]</sup> reported the coefficient of determination ( $r^2$ ).<sup>[52]</sup> Also there are variations in the way correlation coefficient has been determined. Leonardi et al.<sup>[51]</sup> and Seetharaman et al.<sup>[52]</sup> compared results of the response versus nominal concentration and this was what was done for the current study, while Hung et al and Luebke et al. reported by comparing results of the standard method to those of the proposed method.

Limit of detection and limit of quantitation parameters were not viewed as essential by other researchers and were not determined when validating RCP analytical methods<sup>[51, 52]</sup>. This was because the high counts that are used for patient doses are not affected by background activity. Also, due to the spontaneous and random radioactivity, specific readings could only be taken within a short space of time. The current study however identified the need to determine these parameters. A sample reading or a measurement taken using radiation detection equipment like most analytical chemistry equipment consists of a signal which provides the required information about the analyte, and noise which are simply fluctuations of background activity which are unwanted<sup>[60]</sup>. For patient doses, noise tends not to affect the

high counts that are recorded. However in RCP analysis, where tests involve use of low counts, background activity tends to interfere with the reading, limiting the accuracy and precision of the measurement. But despite these shortfalls, there was still a need to be able to obtain an accurate reading that would not be affected by background interference or noise. Results of the current study that were obtained after taking counts of blank samples and determining the standard deviation showed the same number of counts for the detection limit for all three chromatography strips, despite the difference in the total lengths as well as specific regions of interest on each strip. The results showed that it was possible to determine an accurate reading and this would be a guide for any analyst performing the analytical test. The validation protocol template in the addendum B can assist in the design of a validation protocol for the radiochemical purity analysis done with TLC, ITLC or paper chromatography.

## CONCLUSION

The aim of the study was to investigate if a RCP test method can be validated based on ICH Q2A and Q2B guidelines using facilities and equipment available in a public hospital radiopharmacy and to establish a sample method validation protocol which can also be applied to other radiopharmaceuticals. The study involved validation of two alternative RCP analytical test methods for Tc-99m sestamibi that have already been incorporated in routine practice against a method similar to the manufacturer's prescribed analytical method.

From the findings, the conclusion is that all the 8 parameters are essential. Some kind of LOD and LOQ determination is essential as they can guide the analyst on how much activity should be added on the strip in order to have accurate results. Also, validating an analytical procedure in a hospital setting is possible once some important prerequisites or requirements are met. There is a need for trained staff preferably in radiopharmacy or radiochemistry that can correctly analyse the results. There is a need for the correct specifications of the materials in order to undertake the validation process. Reference standards have to be prepared in house due to the short half-life of Tc-99m. However, for radiopharmaceuticals that are documented to have other impurities other than free pertechnetate and hydrolysed reduced technetium, it could be important to have the HPLC equipment available in order to identify any additional impurities which might not be identified by the paper or TLC chromatographic method.

In order to assist radiopharmacy staff to perform method validation, a template protocol is proposed in Addendum B. For hospital pharmacies that meet the basic requirements needed in order to validate a RCP analytical method, the template will be able to provide a standardized procedure for undertaking the exercise while at the same time provide the opportunity for any analyst to adapt the validation plan to their specific settings.

**ADDENDUM A****Table A<sub>1</sub>: Results of each radiochemical component (Tc-99m sestamibi, colloid and pertechnetate) individually tested on each chromatographic strip**

Sample	MN Alox		Whatman 31ET		Schleicher and Schuell	
	A %impurity	A LE(%)	W %impurity	W LE(%)	S %impurity	S LE(%)
Tc-99m MIBI	0.20	99.80	0.50	99.40	0.40	99.50
	0.10	99.80	0.40	99.50	0.50	99.40
	0.10	99.80	0.40	99.50	0.50	99.20
	0.10	99.80	0.50	99.50	0.60	99.20
Tc-99m colloid	99.80	0.20	99.70	0.10	99.70	0.20
	99.80	0.20	99.50	0.10	99.70	0.20
	99.90	0.10	99.50	0.10	99.70	0.20
	99.70	0.20	99.50	0.10	99.40	0.40
Tc-99m pertechnetate	4.50	94.60	99.40	0.40	87.10	12.40
	6.50	92.10	99.00	0.80	97.20	1.00
	11.00	87.90	99.10	0.40	97.80	0.70
	8.50	89.70	99.30	0.60	96.30	2.70

The average percentages and standard deviation of each radiochemical component in the lower and upper ROIs shown in Figures 1 and 2 are outlined below. All values are for four samples.

For the lower ROI (Figure 1):

1. Tc-99m MIBI: A; 0.1 %  $\pm$  0.1%, W; 0.5 %  $\pm$  0.1 % and S; 0.5%  $\pm$  0.1 %.
2. Tc-99m Colloid: A; 99.8 %  $\pm$  0.1 %, W; 99.6 %  $\pm$  0.1 % and S; 99.6 %  $\pm$  0.1 %.
3. Pertechnetate; A; 7.6 %  $\pm$  2.8 %, W; 99.2 %  $\pm$  0.2 % and S; 94.6 %  $\pm$  5.0 %.

For the upper ROI (Figure 2):

1. Tc-99m MIBI: A; 99.8 %  $\pm$  0.0 %, W; 99.5 %  $\pm$  0.0 % and S; 99.3 %  $\pm$  0.1 %.
2. Tc-99m Colloid: A; 0.2 %  $\pm$  0.1 %, W; 0.1 %  $\pm$  0.0 % and S; 0.3 %  $\pm$  0.1 %.
3. Pertechnetate: A; 91.1 %  $\pm$  2.9 %, W; 0.6 %  $\pm$  0.2 % and S; 4.2 %  $\pm$  5.5 %.



**Table A<sub>2</sub>: Calculated (nominal) values of Tc-99m sestamibi, colloid and pertechnetate**

<b>Aimed for (%)</b>	<b>sestamibi (%)</b>	<b>colloid (%)</b>	<b>pertechnetate (%)</b>
80	80.8	9.5	9.7
85	85.6	7.1	7.3
90	90.4	4.7	4.8
95	95.2	2.4	2.4

**Table A<sub>3</sub>: Results of samples containing varying concentrations of Tc-99m sestamibi**

Sample No.	Nominal	A LE (%)	W LE (%)	S LE (%)
	Concentration			
<b>1</b>	<b>80.8 % sestamibi</b>	89.3	84.9	85.6
<b>2</b>		90.5	84.5	87.3
<b>3</b>		94.7	87.9	87.1
<b>4</b>		94.2	85.5	84.9
<b>5</b>	<b>85.6 % sestamibi</b>	95.2	88.4	91.2
<b>6</b>		95.5	89.0	91.1
<b>7</b>		97.0	91.6	91.1
<b>8</b>		97.0	92.4	91.9
<b>9</b>	<b>90.4 % sestamibi</b>	97.4	94.7	95.4
<b>10</b>		98.0	94.4	95.4
<b>11</b>		98.4	95.7	95.5
<b>12</b>		98.2	96.8	96.2
<b>13</b>	<b>95.2 % sestamibi</b>	99.4	98.2	97.8
<b>14</b>		99.4	98.5	98.4
<b>15</b>		99.5	98.5	98.6
<b>16</b>		99.6	98.2	98.0
<b>17</b>	<b>100% sestamibi</b>	99.8	99.4	99.5
<b>18</b>		99.8	99.5	99.4
<b>19</b>		99.8	99.5	99.2
<b>20</b>		99.8	99.5	99.2

The mean measured concentrations and the mean standard deviations of the varying calculated (nominal) concentrations of Tc-99m sestamibi are outlined below.

1. 80.8 % MIBI: A; 92.2 %  $\pm$  2.7 %, W; 85.7 %  $\pm$  1.5 %, S; 86.2 %  $\pm$  1.2 %.
2. 85.6 % MIBI: A; 96.2 %  $\pm$  1.0 %, W; 90.4 %  $\pm$  2.0 %, S; 91.3 %  $\pm$  0.4 %.
3. 90.4 % MIBI: A; 98.0 %  $\pm$  0.4 %, W; 95.4 %  $\pm$  1.0 %, S; 95.6 %  $\pm$  0.4 %.
4. 95.2 % MIBI: A; 99.5 %  $\pm$  0.0 %, W; 98.4 %  $\pm$  0.2 %, S; 98.2 %  $\pm$  0.4 %.
5. 100 % MIBI: A; 99.8 %  $\pm$  0.0 %, W; 99.5 %  $\pm$  0.1 %, S; 99.3 %  $\pm$  0.2 %.

**Table A<sub>4</sub>: Results of blank scans**

scan no	Whatman 31ET			Schleicher and Schuell			Aluminium oxide		
	counts W1	counts W2	total counts	counts S1	counts S2	total counts	counts Al1	counts Al2	total counts
1	9	22	34	6	23	34	7	22	34
2	13	24	44	10	20	44	11	19	44
3	17	25	49	13	24	49	14	23	49
4	7	24	39	6	20	39	7	19	39
5	15	19	41	12	17	41	13	16	41
6	6	19	30	5	16	30	5	15	30
7	14	17	37	10	16	37	11	15	37
8	10	22	35	7	17	35	8	16	35
9	13	31	50	10	26	50	11	25	50
10	11	20	37	9	19	37	10	18	37
11	8	11	27	6	12	27	7	11	27
12	13	25	46	9	22	46	10	21	46
13	15	22	39	12	19	39	13	18	39
14	14	17	34	11	16	34	12	15	34
15	9	14	29	8	10	29	9	10	29
16	5	22	32	4	18	32	4	17	32
17	16	18	40	11	16	40	12	15	40
18	13	14	37	10	15	37	11	14	37
19	16	18	39	13	14	39	14	13	39
20	23	21	51	18	18	51	20	17	51

W1 – Counts in lower ROI on Whatman 31ET chromatography strip.

W2 – Counts in upper ROI on Whatman 31ET chromatography strip.

S1 – Counts in lower ROI on the Schleicher and Schuell chromatography strip.

S2 – Counts in the upper ROI on the Schleicher and Schuell chromatography strip.

Al1 – Counts in the lower ROI on the MN Alox TLC strip.

Al2 – Counts in the upper ROI on the MN Alox TLC strip.

**Table A<sub>5</sub>: Comparison of inter-personnel variation**

Sample No.	Analyst A			Analyst B		
	M LE (%)	W LE (%)	S LE (%)	M LE (%)	W LE (%)	S LE (%)
1	99.8	99.4	99.5	99.8	99.6	99.5
2	99.8	99.5	99.4	99.8	99.8	99.2
3	99.8	99.5	99.2	99.8	99.8	99.1
4	99.8	99.5	99.2	99.7	99.7	99.4
5	99.8	93.4	99.0	99.8	99.5	99.4
6	99.6	97.1	97.4	99.7	99.5	99.1
7	99.8	97.9	98.8	99.8	99.6	98.4
8	99.8	99.9	99.1	99.7	99.4	99.3

The average labeling efficiencies and mean standard deviations of the effect of inter personnel variations are outlined below.

Analyst A: A; 99.8 %  $\pm$  0.1 %, W; 98.3 %  $\pm$  2.2 %, S; 99.0 %  $\pm$  0.7 %.

Analyst B: A; 99.8 %  $\pm$  0.1 %, W; 99.6 %  $\pm$  0.2 %, S; 99.2 %  $\pm$  0.4 %.

**Table A<sub>6</sub>: Results of the effect of lag time**

Sample No.	Group 1 immediate			Group 2 with lag time		
	MLE	W LE	S LE	MLE	W LE	S LE
1	99.8	99.4	99.5	99.7	99.5	99.3
2	99.8	99.5	99.4	99.7	98.1	98.0
3	99.8	99.5	99.2	99.7	99.3	99.1
4	99.8	99.5	99.2	99.5	98.8	98.8
5	99.8	93.4	99.0	99.7	99.3	98.6
6	99.6	97.1	97.4	99.5	98.7	99.2
7	99.8	97.9	98.8	99.8	99.6	99.5
8	99.8	99.9	99.1	99.7	99.3	99.2
9	99.8	99.6	99.5	99.7	99.7	99.8
10	99.8	99.8	99.2	99.8	99.6	99.4
11	99.8	99.8	99.1	99.8	99.6	99.3
12	99.7	99.7	99.4	99.7	99.7	99.5
13	99.8	99.5	99.4	99.8	99.7	99.4
14	99.7	99.5	99.1	99.8	99.7	99.5
15	99.8	99.6	98.4	99.8	99.7	99.1
16	99.7	99.4	99.3			

The average labeling efficiencies and mean standard deviation of immediate and 2 minute lag time are outlined below.

Immediate: A; 99.8 %  $\pm$  0.1 %, W; 98.9 %  $\pm$  1.7 %, S; 99.1 %  $\pm$  0.5 %.

2 minute lag time: A; 99.7 %  $\pm$  0.1 %, W; 99.4 %  $\pm$  0.5 %, S; 99.2 %  $\pm$  0.4 %.

## ADDENDUM B

### VALIDATION PROTOCOL

Protocol for validation of radiochemical purity analysis of [radiopharmaceutical] prepared by  
[hospital radiopharmacy]

Protocol No.: .....

Analyst:

.....

Date: .....

Radiopharmacist / Qualified person in charge:

.....

Date: .....

Edited by:

.....

Date: .....

Approved by:

.....

Date: .....

Issue date: .....

## OBJECTIVE

To validate analytical procedures for radiochemical purity of [radiopharmaceutical] prepared by [hospital radiopharmacy].

## SCOPE

The scope of this protocol covers the validation of analytical procedures for radiochemical purity of [radiopharmaceutical] to be used at [name of institution].

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## **RESPONSIBILITIES**

Preparation of validation protocol	Radiopharmacist / Qualified person in charge
Performing validation exercise	Radiopharmacist / analyst assigned to radiopharmacy
Analysis of acquired data	Radiopharmacist / Qualified person in charge

## **VALIDATION PARAMETERS OR CHARACTERISTICS**

The following analytical performance characteristics will be determined during the validation

1. Specificity
2. Accuracy
3. Repeatability and intermediate precision
4. Linearity and range
5. Limit of detection and Limit of quantitation of [equipment]
6. Robustness



## MATERIALS AND METHODS

Below are materials that are required when validating radiochemical purity analysis

### 1. Materials for reference analytical method

- Stationary phase materials prepared to specification
- Mobile phase
- Procedure for radiochemical purity determination

### 2. Alternative analytical method materials

- Stationary phase prepared to specification
- Mobile phase
- Procedure for radiochemical purity determination

### 3. Radiopharmaceutical to be analysed

### 4. Standards

### 5. Calibrated standard of radiopharmaceutical if available

### 6. Standards of impurities e.g. Tc-99m pertechnetate

### 7. Equipment

- Gamma counting equipment (for example dose calibrator, scintillation well counter, radiochromatogram scanner, gamma camera)

**Note:** If the equipment does not provide information of the distribution of radioactivity over the length of the chromatography strip, the strip can be cut into short sections, which can then be individually counted in a gamma counting equipment and a plot can be constructed.

- Other equipment (e. g. oven, water bath, centrifuge)

## **SAFETY PRECAUTION**

Personnel handling radioactive substances need to wear appropriate protective clothing, work with appropriate radiation shielding equipment and follow general radiation protection principles.

## **ANALYTICAL PERFORMANCE CHARACTERISTICS TO BE EVALUATED**

### **1. Specificity**

#### **Definition and description of specificity**

Specificity is the ability to assess unequivocally the radiopharmaceutical compound in the presence of other radiochemical components which may be expected to be present. This investigation is carried out to identify and quantify the radiopharmaceutical compound and also to accurately determine the impurities that might be present

#### **Test procedure to determine specificity**

To determine specificity, first test the radiopharmaceutical compound and each expected impurity individually in order to identify the location of peaks on the chromatographic strips. Then prepare samples containing known amounts of the radiopharmaceutical compound and the expected impurities and test to determine whether the analytical procedure is able to separate the individual components present in the sample.

Perform 3 analyses for each radiochemical component (radiopharmaceutical compound and expected impurities) individually on the chromatographic strip.

Perform 3 analyses of a sample containing known concentration of the radiopharmaceutical compound and known concentrations of impurities.

#### **Acceptance criteria**

Ability to separate and show components present in the sample

Compare the  $R_f$  values of the radiopharmaceutical compound and each impurity to verify that there is no overlap of peaks i.e. no co-elution of impurities with the radiopharmaceutical compound

## 2. Accuracy

### Definition and description of accuracy

Accuracy expresses the closeness of agreement between the true value and the value found in analysis. This investigation helps to show the extent to which the tests results and the true value agree.

#### Test procedure to determine accuracy

Perform 9 determinations (3 replicates) over 3 concentrations over a range ( $90\% \pm 10\%$  or pharmacopoeial labeling efficiency limit  $\pm 10\%$ ). Calculate the mean result for each concentration. Compare results obtained of the alternative analytical method/s to those obtained with the pharmacopoeial or manufacturer's method or known values of a standard.

#### Acceptance criteria

The mean of the measured values for alternative analytical method should not differ more than 2 % from the mean value of the recommended analytical procedure or known value of the standard.

## 3. Precision

### Definition and description of precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions.

For **repeatability**, tests are carried out in the same laboratory within short intervals of time and by the same analyst using the same equipment. (Analyst to ensure that analysis is performed within the shelf life of the radiopharmaceutical)

**Intermediate** precision typically involves tests that are performed in the same laboratory but on different days, by different analysts and using different counting equipment (e.g. dose calibrator, scintillation well counter, gamma camera) if applicable.

**Test procedure to determine repeatability precision**

Perform 6 analyses at 100 % test concentrations or 9 determinations from accuracy data.

Calculate the average and the percentage relative standard deviation (% RSD) for the results obtained.

Formula for % RSD is:

$$(\text{SD/average}) \times 100$$

**Acceptance criteria**

Percentage relative standard deviation (% RSD) should be 15 % or less.

**Test procedure to determine intermediate precision**

Perform 2 x 6 analyses at 100 % test concentrations, e.g. on different days or by different operators.

Calculate the average for each set of results and determine the percentage relative standard deviation (% RSD) for both sets.

Perform T-test for independent samples to compare the two groups.

**Acceptance criteria**

Percentage relative standard deviation (% RSD) should be 15 % or less.

For Student T-tests for independent samples,  $p < 0.05$ .

**4. Linearity and range****Definition and description of linearity**

Linearity is the ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of radiopharmaceutical compound in the sample. For radiopharmaceuticals, samples containing varying concentrations of the radiopharmaceutical compound and impurities are used, usually containing decreasing

concentration of the radiopharmaceutical compound with corresponding increasing concentrations of impurities.

### **Test procedure to determine linearity**

Perform analyses on a minimum of 5 concentrations (with a range  $90\% \pm 10\%$  or pharmacopoeial labeling efficiency limit  $\pm 10\%$ ) and perform linear regression analysis.

### **Acceptance criteria**

Correlation coefficient (r) should be  $\geq 0.98$ .

Y-intercept should be  $\leq 5\%$  of the target concentration

Slope should be 1

## **5. Limit of detection and limit of quantitation**

### **Definition and description of limit of detection**

The detection limit is the lowest amount of activity that a radiation detecting equipment can distinguish from background activity. This then is the point at which the value of the radiopharmaceutical compound is larger than the uncertainty associated with the counting method. Limit of quantitation is the lowest amount of radioactivity in a sample which can be quantitatively determined with that equipment with suitable precision and accuracy.

### **Test procedure to determine the limit of detection and limit of quantitation**

Perform twenty blank scans performed using the chromatographic strips for the alternative and the recommended analytical procedures.

Calculate standard deviation of the blank samples.

### **Acceptance criteria**

Limit of detection is three times the standard deviation of blank samples.

Quantitation is three times the detection limit

## **6. Robustness**

### **Definition and description of robustness**

Robustness is a measure of the method's capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. The information acquired from robustness tests can guide on the specific conditions under which the analytical procedure needs to be carried out and if need be a precautionary statement can be included in the procedure. Examples of variations that are noted in hospital radiopharmacy include storage of stationary and mobile phases, drop size, lag time, type of developing chambers used and inter personnel variation.

### **Test procedure to determine robustness**

From the same sample;

Perform 6 determinations at 100 % test concentrations (control) and

Perform 6 determinations from the same sample where one of the variations is applied to the analytical procedure.

Calculate the average and the percentage relative standard deviation (% RSD) for each set of results obtained.

Perform T-test for independent samples to compare the results of the variation with results of repeatable precision (control).

### **Acceptance criteria**

Percentage relative standard deviation (% RSD) should be 15 % or less.

For Student's T-tests for independent samples,  $p < 0.05$ .

## VALIDATION REPORT

After completion of the methods validation experiments, the results should be analysed and evaluated after which a final report has to be issued.

The basic contents of the report can include;

1. Purpose / objectives
2. Scope
3. Responsibilities
4. References
5. Safety Precautions
6. Materials
  - Reagents that were used
  - Reference standards that were used
  - Equipment that were used
  - Describing the parameters that were used to validate the analytical procedure
7. Methodology and results
  - Explain how the samples were prepared
  - How each parameter was determined
  - Show representative plots, chromatograms (raw data)
  - Method acceptance criteria that was used
  - Statistical procedures and representative calculations
8. A discussion of the results and a conclusion on the acceptability of the alternative method that was validated

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